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Protein Purification

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This monograph summarizes recent developments in the purification and analysis of natural and recombinant proteins. The basic strategies employed in protein purification are reviewed with regards to the characteristics of the protein of interest that may aid its isolation, choice of the starting material, and use of denaturants. Preparation of cell-free extracts followed by bulk precipitation and/or phase partition constitute the initial steps of many purification schemes. Chromatographic methods (size exclusion, ion exchange, hydroxylapatite, reversed phase, hydrophobic interaction and affinity based) utilizing either traditional, low pressure or high-performance liquid chromatography instrumentation are discussed. Electrophoretic techniques used to analyze the homogeneity of the protein product include SDS-PAGE, isoelectric focusing, IEF and two dimensional gel electrophoresis.

Abbreviations

a, selectivity factor; ABx, antibody exchanger; cDNA, complementary DNA; CM, carboxymethyl; CoA, coenzyme A; D, diameter; Da, Daltons; DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; FPLC, fast protein liquid chromatography; FR, flow rate; HETP, height equivalent to a theoretical plate; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; IgG, immunoglobulin G; IgM, immunoglobulin M; K, distribution coefficient; k', capacity factor; L, length; LV, linear velocity; N, number of theoretical plates; PAGE, polyacrylamide gel electrophoresis; PEI, polyethyleneimine; PEG, polyethylene glycol; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; QAE, quaternary aminoethyl; R, resolution; RNA, ribonucleic acid; Rt, retention time; SDS, sodium dodecyl sulfate; SF, scale-up factor; SP, sulfoxypropyl; TFA, trifluoroacetic acid; V, retention volume; V₀, void volume.

1 Introduction—Historical Perspective

Early methods of protein separations date to the beginning of the current century. Analytical separations using electrophoretic techniques were developed shortly after electric power became commercially available. These were simply performed in liquid media filling a U-shaped glass tube with the electrodes suspended in each of the open ends. Early methods of preparative-scale protein isolations relied upon their solubility characteristics. Prior to the 1940's, acidic, organic and salt solutions were used to precipitate proteins from complex mixtures of biological fluids. However, as solubility-based purification methods were refined, it became increasingly apparent that not only were these relatively crude in their ability to produce a homogeneous protein preparation but also often produced a preparation devoid of biological activity. During the 1940's, ultracentrifugation appeared as a powerful new tool that offered the ability to separate proteins on the basis of size rather than solubility characteristics. Although biological activity was often retained after such a separation, it was several years before ultracentrifugation was applied to preparative protein isolations. Electrophoretic methods of protein separations were also improved during this period. Liquid media utilized in earlier times were replaced by various types of

solid supports (e.g. paper, starch, agar and polyacrylamide). These offered advantageous properties of mechanical rigidity as well as chemical inertness that improved analytical resolution of protein mixtures.

The next two decades witnessed the development of two new chromatographic techniques that revolutionized the field of protein chemistry. Initially, ion exchange and later size exclusion chromatography were introduced as novel, preparative-scale techniques based upon protein charge and size, respectively. Methods of electrophoresis were also refined, by the use of discontinuous pH conditions and addition of a variety of modifying agents, particularly detergents (e.g. sodium dodecyl sulfate). The utility that both of the new chromatographic and electrophoretic methods offered to the protein chemistry laboratory is verified by their continued popularity today. Two important developments during the 1960's and 1970's greatly enhanced the field of protein purification. Affinity chromatography, appearing in the latter part of the 1960's, allowed the isolation of proteins based on their biological rather than physicochemical characteristics. This was a particularly important development since the number of different physical or chemical properties upon which to base separations is limited. It is not uncommon to achieve an operationally complete purification of a protein from crude starting material using a single affinity-based separation protocol as, for example, the purification of immunoglobulin G from serum using *Staphylococcus aureus* protein A bound to a solid support¹¹. The second development, high-performance liquid chromatography (HPLC), introduced in the late 1970's, has not only led to second generation application of established techniques of conventional liquid chromatography (ion exchange, size exclusion and affinity chromatography) but also the direct development of new technologies (reversed phase and hydrophobic interaction chromatography). The future of protein separations will continue to rely on the application of new products (e.g. fluoropolymers) and developments to chromatography and electrophoresis such as radial flow, supercritical fluid and open tubular liquid chromatography and capillary electrophoresis.

The following monograph is an attempt to summarize the state of the art of protein purification. We have directed this review primarily toward those individuals who are new to the field and desire a concise treatise from which they can refer to the original literature for potential application to their own problem. Although we have attempted to summarize as many aspects of protein purification as possible, the reader is advised that it is impractical to cover such a broad topic (on which entire texts have been written) without inadvertently omitting some areas. Thus, the references cited herein are intended only to serve as examples and should not be considered as inclusive. In most instances, these references have been limited to the past four to five years to provide the reader with the most up-to-date developments. Other extensive reviews have recently appeared and may be consulted should further assistance be necessary. Several books have been published that deal exclusively with both theoretical and practical aspects of protein purification²⁻⁵. Others have appeared in the scientific literature as compilations⁶ or general review articles⁷⁻¹⁰ or specifically limited to the purification of selected proteins such as peptide hormones¹⁹, growth factors²⁰, enzymes^{21,22} or proteins of plant^{23,24}, viral²⁵ or membrane²⁶ origin. Specific types of separation methods have also been summarized including size exclusion^{27,28}, ion exchange²⁹, hydrophobic interaction³⁰ and affinity³¹⁻³⁴ chromatography.

2 Development of a Protein Purification Strategy

2.1 General Considerations

Three basic steps are performed when attempting to isolate a protein. First, small-scale, analytical experiments are used to analyze the feasibility of a particular separation technique on the protein of interest. These are organized in a sequence such that the end product is free of contaminating proteins. Second, the process is increased to a preparative-scale allowing sufficient material to be isolated at the end of the process. Finally, the purity of the isolated protein is checked. Often it may be necessary to amend the purification scheme based upon the final degree of purity since contaminants may appear during the large-scale purification that were not detected during the small-scale experiments. In this case it is convenient to analyze a small amount of material from the last stage of the preparative-scale separation scheme, scale up to the preparative mode if satisfactory and reexamine for purity. Thus, one can readily appreciate the trial-and-error process that must usually be performed to achieve an end product of acceptable purity. It is important to emphasize that the concept of "acceptable purity" varies with the protein at hand. For example, the purity of a protein with demonstrable biological activity such as an enzyme may simply mean freedom from inhibitors or interfering substances. On the other hand, purification of structural proteins often requires evidence of the absence of all detectable contaminants.

2.2 Sample and Methods Characteristics Influencing Purification

There are several factors to be considered when the initial small-scale purification strategy is conceived. Foremost among these is an understanding of the biological and physicochemical properties of the protein one is attempting to isolate. Does it possess a highly specific ligand that could be exploited in an affinity-based separation protocol? Do its charge, molecular weight, isoelectric point and/or hydrophobicity lend themselves to a particular isolation technique that should be undertaken early in the purification scheme? A second factor to consider is the objective for purification. To retain biological activity it is normally required that all separation techniques be compatible with maintaining secondary, tertiary and quaternary structure. Limitation to nondenaturing conditions to preserve protein conformation restricts one's choice of techniques that can be employed. On the contrary, if biological activity needs not be preserved, as for example when the objective is to prepare a protein suitable for amino acid sequence analysis, the list of potential separation techniques includes those that are denaturing as well as nondenaturing.

Independence of the methods chosen also affects the efficacy of a particular purification strategy. A common practice is to sequentially use separation techniques founded upon different physicochemical properties and which maximize the greatest differences between the physical properties of the protein of interest and its contaminants. In this regard, affinity chromatography, if available, should be considered in every purification scheme since it is the only commonly available technique that

is based on the biological rather than physical properties of proteins. Affinity-based separation methods that rely on the antigenic characteristics of proteins should be considered in the same light. Finally, the actual sequence of steps one chooses may greatly affect the final yield and purity of the end product. It is a general rule of thumb to utilize a different mode of separation at each succeeding step³⁵. Figure 1 illustrates

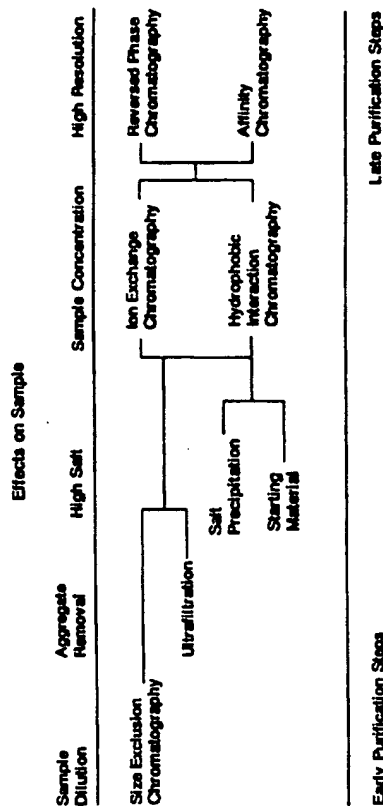


Fig. 1. The sequence of purification steps and their effects on sample conditions are illustrated as a guide for designing a purification strategy

the effects various separation techniques have on the protein sample. These effects should be considered with regards to the sequence of the different techniques when designing a protein purification strategy. Diluting procedures such as ultrafiltration and size exclusion chromatography are not only effective in removing aggregates that may interfere with subsequent chromatographic steps but also minimize the effects of proteases that may be present in the starting material. In some cases, therefore, these procedures may prove to be beneficial if used early in a purification scheme. High resolution techniques such as reversed phase and affinity chromatography can then be employed later to achieve final purity. Occasionally, however, concentrating methods (ion exchange, reversed phase, hydrophobic interaction and affinity chromatography) may be more appropriate at an initial stage particularly if the protein one wishes to purify is present at a low concentration and/or in a large volume of beginning material. Proteolysis can then often be substantially reduced by addition of appropriate inhibitors (see below).

Another factor to be considered when designing the order of steps is the condition in which the protein emerges from a particular step. Hydrophobic interaction chromatography may be a useful fractionation procedure early in a purification scheme when the sample is in a high ionic strength aqueous solution, for example following ammonium sulfate precipitation or isolation from a culture medium containing high levels of salts³⁶. Reversed phase or affinity chromatography following hydrophobic interaction chromatography can then be used to both desalt and further purify

the protein of interest³⁷). However, desalting steps which do not result in further sample purification should be avoided since they are time-consuming and often lead to an overall reduction in final yield. Indeed, when performing sequential purification steps utilizing differential ionic strength buffers, it may be worthwhile to experiment with dilution of the eluting salt concentration or direct loading of a fraction from a previous step without desalting. Vardanis³⁸, for example, observed increased resolution by sequential ion exchange chromatography when the sample was reappplied in the eluting salt from the previous column as compared to when the sample was first desalted. Similarly, a 50% increase in purification of glucose-6-phosphate dehydrogenase was achieved by anion exchange chromatography when the enzyme was applied in an aqueous buffer containing 0.38 M NaCl compared to the same buffer devoid of salt³⁹.

2.3 Assessment of Purity

An integral part of developing a protein purification strategy is the availability of a monitoring system to follow the elution profile of the protein from a given chromatographic column. For proteins with known biological activity, specific assays are simply utilized to follow the purification. Proteins with no recognized activity, for example structural proteins, require an alternative strategy for their identification. The availability of an antibody, either monoclonal or polyclonal, to such proteins allows the use of a detection system based upon the antigenic properties of the protein. However, in this case one often finds oneself in a circular set of circumstances whereby an antiserum cannot be prepared without the purified protein and purification cannot be achieved without an antiserum. One recent advance that begins to break this circle is the use of antisera prepared against synthetic peptides whose amino acid sequence is determined from the nucleotide sequence of the corresponding complementary DNA (cDNA) or genomic DNA. However, once again this may be inappropriate since it is often the objective of the purification scheme to prepare a protein for amino acid sequence analysis from which synthetic oligonucleotide probes are generated to isolate the corresponding gene.

The assessment of protein homogeneity occupies a cornerstone in the purification strategy not only for its ability to analyze the purity of the final product but also for its role in developing a monitoring system. Traditionally, electrophoretic techniques have complemented chromatography as the method of choice for assessing purity. One-dimensional discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) is a powerful method of resolving complex protein mixtures on the basis of their molecular weight. Isoelectric focusing (IEF) separates proteins on the basis of their net charge. Two-dimensional gel electrophoresis was developed to increase the resolving power of conventional electrophoresis by first separating proteins according to their net charge on IEF gels followed by SDS-PAGE. Once separated, proteins are visualized by Coomassie blue or silver staining. Combined two-dimensional SDS-PAGE followed by silver staining has the potential to separate and identify over 100 different proteins on a single gel⁴⁰. Indeed, the high resolving power of two-dimensional gel electrophoresis makes it an ideal choice as a detection system for a protein purification strategy. Anderson and

Anderson⁴¹ have advocated a rational approach to protein fractionation whereby the starting material is subjected to a variety of different small-scale analytical procedures from which the protein of interest is identified on two-dimensional gels (Fig. 2). The procedure that produces the greatest molecular weight and charge separation from contaminants can then be scaled up to a preparative mode followed by subsequent separations according to size and/or charge parameters theoretically yielding a pure product after a three- or four-step protocol. As technological developments continually reduce the amount of protein required for visualization, these electrophoretic methods will occupy a larger role in the future of protein purification.

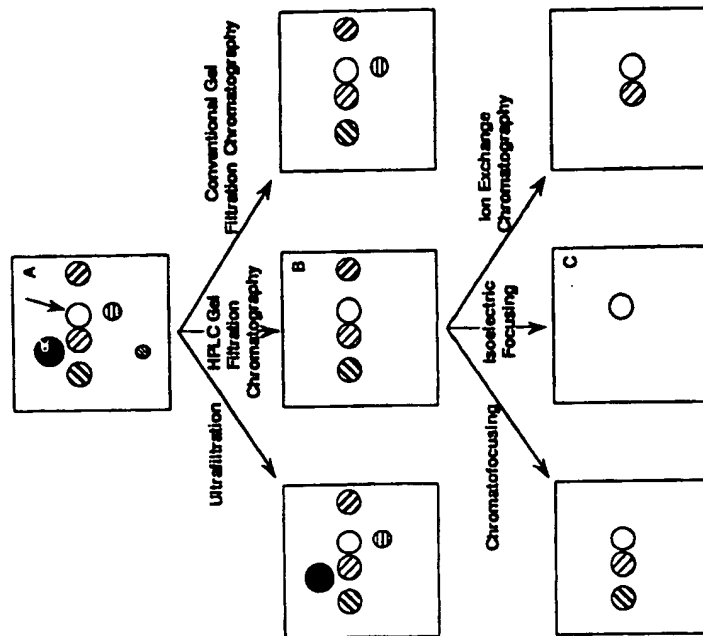


Fig. 2. Designing a rational approach to protein purification⁴¹. In this hypothetical example, two dimensional polyacrylamide gel electrophoresis of the starting material (A) reveals a large array of proteins in addition to the component of interest (indicated by the arrow). A variety of small-scale molecular weight separation techniques are tested and HPLC gel filtration chromatography is found to be most effective in removing contaminants (B). Several analytical charge separation techniques are then tested of which isoelectric focusing is found to produce purification to homogeneity (C). In this case, the final purification scheme would be increased to preparative scale HPLC gel filtration and isoelectric focusing.

3 Choice of Starting Material

3.1 Relative Protein Abundance

Deciding which initial steps to use in a purification scheme depends upon both the concentration and physical state in which the protein exists in the starting material. Proteins initially present at relatively high concentrations in the starting material are normally easily purified. For example, Fox et al.⁴² employed a four-step purification scheme consisting of DEAE-Sephadex chromatography, ammonium sulfate precipitation, phenyl-Sepharose and ion exchange chromatography to isolate an enzyme constituting 2.5% of the total soluble protein of *Methanobacterium thermoautotrophicum*. Proteins present at only trace amounts, on the contrary, are often extremely difficult to purify particularly if a contaminant is present in the starting mixture at a relatively high percentage of the total protein content. However, the same component of interest when present at a higher or equal abundance in a different source (e.g. different tissue) that contains a large variety of constituents but none at a high concentration may be easier to purify. Three examples can be cited to illustrate this point. First, preparative-scale isolation of the human interferon- γ receptor was achieved after identification of placenta as a crude starting material that expressed greater than 30 times the amount of receptor compared to cells grown in tissue culture⁴³. Second, the envelope glycoprotein of human T-cell leukemia virus type I was purified to homogeneity using virus infected cell extracts rather than purified virions as starting material⁴⁴. Although this virus contains less than 15 proteins compared to the thousands present in its host cells, it was found to be a poorer source for isolating the envelope protein in part because this protein dissociates from the virus during density gradient centrifugation and was therefore present at a relatively lower amount compared to infected cells. Finally, purification of the low molecular weight rabbit plasma protein α_1 -microglobulin was aided by the use of nephrotoxic chemicals⁴⁵. Renal tubular damage induced by sodium chromate caused a low molecular weight proteinuria characterized by the absence of larger plasma proteins (e.g. albumin and immunoglobulins) and relatively enriched for α_1 -microglobulin thus facilitating its isolation.

In an analogous vein, the isolation and characterization of novel biologically active polypeptides and proteins has recently been achieved with the advent of serum-free tissue culture media. Two examples are noteworthy. First, serum-free media have provided assay systems used to monitor the purification of a variety of protein growth factors without interference from endogenous factors present in serum. MTW9/PL2 rat mammary tumor cells maintained in serum-free culture were used to isolate a growth promoting activity from porcine pituitary extracts which was subsequently identified as transferrin⁴⁶. Lactoferrin was similarly purified and found to possess mitogenic properties for human lymphocytes⁴⁷. These reports complement other examples of purification of new tissue culture derived growth factors, including those derived from epidermal⁴⁸, platelet⁴⁹ and fibroblast⁵⁰ cell culture. Secondly, other cytokines present at nanomolar to picomolar concentrations in tissue culture media were purified after the development of serum-free culture conditions. Examples of these include a variety of interleukins^{51,52}, colony stimulating factors

51,53), tumor necrosis factor⁵⁰ and a leukemia cell-derived factor⁵⁴. It is therefore apparent that any procedure to enrich for a particular protein in the starting material can greatly enhance the ability to achieve its final purification.

3.2 Physical State of the Protein

The physical state in which the protein of interest exists in the original material also influences the initial purification strategy. Soluble proteins naturally found in biological fluids such as plasma, urine and saliva or excreted by cells into tissue culture media can be directly processed by various separations protocols. In contrast, proteins that are present inside cells or tissues require a preliminary extraction step prior to fractionation. This normally involves physical disruption of the cell by homogenization, sonication or freeze-thawing followed by a slow-speed centrifugation to separate debris from the soluble protein present in the supernatant fluid. Subcellular fractionation can subsequently be achieved by high-speed differential centrifugation to separate the nuclear, mitochondrial, lysosomal, microsomal and soluble components (Fig. 3). Assaying these individual fractions for the presence of the protein of interest often identifies the particular subcellular fraction from which to begin purification although exceptions to this generalization have been reported⁵⁵. Furthermore, membrane-associated proteins may tend to aggregate and exhibit reduced solubility in aqueous buffers. To overcome these problems, a plasma membrane fraction can subsequently be prepared by high-speed centrifugation from which bound proteins can be extracted by the use of detergents⁵⁶. It should be noted, however, that the choice of detergent can often dramatically influence the final protein recovery as illustrated by the studies of Nandi et al.⁵⁹. These authors tested a variety of ionic and nonionic detergents under different extraction conditions and observed that very low concentrations of SDS were most effective in purifying an adenosine triphosphatase from fundic mucosa. Alternatively, some integral membrane plasma proteins have been found to be released into solution by mild proteolysis with a nonspecific protease such as papain which cleaves the hydrophilic extracellular region of the molecule from the lipophilic membrane spanning segment⁶⁰. Preservation of enzymatic activity of human erythrocyte acetylcholinesterase⁶¹ as well as the crystallization and x-ray diffraction studies of human histocompatibility proteins⁶² isolated in this manner have verified the utility of this procedure in producing active and homogenous protein fragments.

It is easier to purify a protein if a good source of it is available. The physiological state and growth conditions of cells affect their level of protein synthesis. It is, therefore, always worthwhile to determine the growth conditions where the rate of synthesis for the protein of interest is the highest and the presence of degradative enzymes (proteases, etc.) is minimal. Generally, the material should be obtained during the early stages of protein synthesis but one should consider that in this case the final yield may be reduced. For example, Fig. 4 illustrates a kinetic analysis of human immunodeficiency virus proteins synthesized following infection of human lymphocytes. In this case, a clear relationship exists between several precursor polypeptides and their normal proteolytic degradation products. The major external glycoprotein, gp160, is converted to a smaller polypeptide, gp120, in a time-dependent manner.

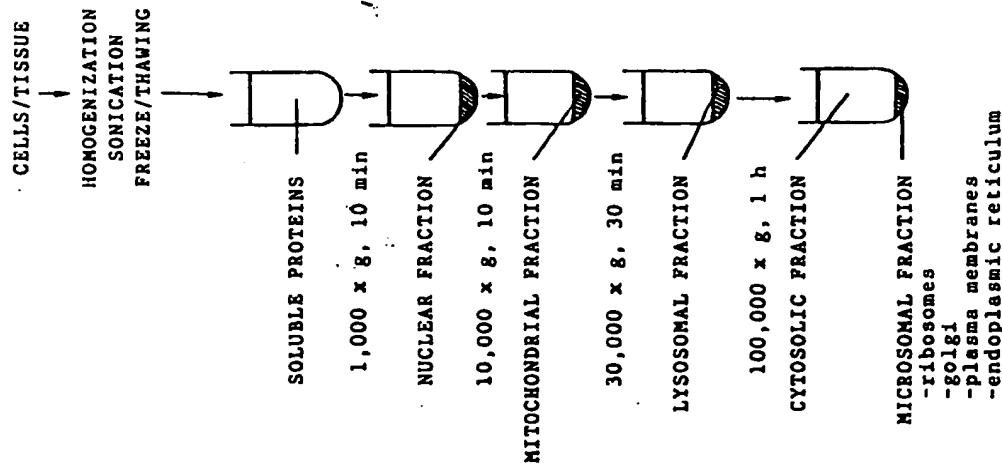


Fig. 3. Differential centrifugation of a cell or tissue extract can be used to fractionate cellular components. Following solubilization of cells and removal of debris by filtration, the soluble proteins can be differentially recovered by centrifugation at successively increasing centrifugal forces

Similarly, the polyprotein p55 is degraded to at least two products (indicated in Fig. 2 as p24) via an intermediate p41 moiety. The purification of gp120 or p24 would therefore be dependent upon the correct kinetic conditions of their respective syntheses.

Individual proteins are degraded at different rates depending on the physiological state of the cell. Damaged and abnormal proteins are quickly degraded in vivo as are many regulatory proteins. Regulation of the rates of synthesis and degradation of metabolically unstable proteins determines their cellular concentration. Adenosine

Protein Purification

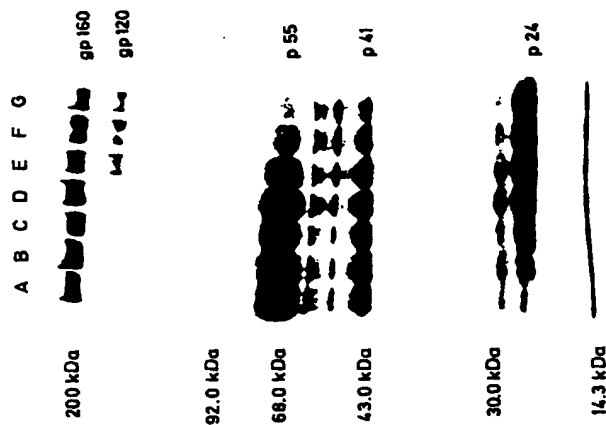


Fig. 4. Kinetic analysis of proteins synthesized by the human immunodeficiency virus type 1 (HIV-1). Human lymphocytes were infected in vitro with HIV-1, labeled for 20 min with [35 S]methionine, resuspended in fresh medium, incubated for various lengths of time, lysed in detergent solution and HIV-1 proteins isolated by immunoprecipitation and analyzed by SDS-PAGE. Lanes A to G illustrate results of 10, 20, 40, 60, 120, 180 and 240 min incubations in fresh medium. The positions of molecular weight markers are indicated on the left in kDa. Viral proteins are indicated on the right

triphosphate dependent intracellular protein turnover involves covalent conjugation of ubiquitin to labile intracellular proteins. Furthermore, the recognition of an amino-terminal residue in a protein appears to mediate its *in vivo* half-life⁶³. The *in vivo* half-life for 6-methylsalicylic acid synthetase, the first enzyme of the patulin pathway, in *Penicillium urticae* was much shorter (7 h) than those of two later enzymes, *m*-hydroxybenzyl alcohol dehydrogenase and isoeopoxydon dehydrogenase (17 and 19 h, respectively). The mechanism of inactivation of many such enzymes involved in secondary metabolism is unknown⁶⁴. Incubation of gramicidin synthetase at 37 °C for 1 h resulted in loss of 85% of its activity. This inactivation in agitated cultures, harvested cells as well as cell-free extracts, was at least partly due to the presence of oxygen⁶⁵. The gramicidin S synthetase complex is present in *Bacillus brevis* at the beginning of idiophase, reaches a peak and then diminishes while cells are producing gramicidin S. A nitrogen overlay to exclude oxygen stabilized the activity for a few hours. This thiol-mediated loss of activity was minimized by maintenance of aerobic conditions.

Several different proteases are present in *P. urticae* during patulin production⁶⁶. 6-Methylsalicylic acid synthetase was stabilized by a combination of NADPH, acetyl coenzyme A (CoA), malonyl-CoA, phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol. Substrates and protease inhibitors stabilized the activity of enzymes involved in penicillin biosynthesis in cell-free extracts^{66,67}. The balance between the rates of synthesis and degradation of these enzymes in secondary metabolism affects their level in the cell. As a result, growth conditions with optimum

mixtures of related compounds of different molecular weight and contain peroxides and carbonyl components which react with protein sulfhydryl groups significantly changing their tertiary structure or activity during purification procedures⁹⁵. Ionic and hydrophobic detergents can be removed from proteins by ion exchange chromatography and hydrophobic interaction chromatography respectively⁹⁷. Dialysis, ultrafiltration, solvent extraction and gel filtration can be employed to remove detergents after dissociation from proteins by using acidic conditions^{96,99}. A strongly bound detergent such as SDS can be competed off by an excess of a weaker detergent. Acetone, ethanol or acid have been used to precipitate proteins out of detergent solutions^{73,100}. Alternatively, organic or acidic solvents can precipitate proteins directly out of solution without the use of detergents^{101,102}.

Many disulfide-containing proteins precipitate in reduced form. Attempts to overcome this problem have involved selective reduction of cystine residues or covalent modification at other amino acids prior to purification. Certain disulfides of human antihemophilic factor were selectively reduced before purification with thiol-disulfide interchange chromatography. Denatured myelin basic protein bound irreversibly to gel filtration media but proteolipid did not¹⁰³. Covalent addition of polyethylene glycol and other hydrophobic agents allowed extraction of these types of proteins into organic solvents¹⁰⁴. Addition of charged groups can also increase the water solubility of certain proteins.

The precipitation or chromatographic behavior of a protein cannot be always predicted by its amino acid sequence. After reduction of disulfide groups, bovine growth hormone is extremely insoluble in water while growth hormones of other species with high sequence homology are soluble. Local surface charges on the proteins which are independent of the net charge could affect the retention of proteins on ion exchangers near the isoelectric point¹⁰⁵. Many proteins have asymmetric surface groups. For example, the DNA binding acidic proteins have a region of basic surface charge which is responsible for their binding to DNA and phosphocellulose⁹¹. Inclusion of denaturants eliminates the surface charge and allows accurate determination of molecular weights by size exclusion chromatography^{68,69,106}.

4.3 Inclusion Bodies

Many eukaryotic proteins accumulate in bacteria as fully reduced and denatured aggregates called inclusion bodies. The presence of inclusion bodies in genetically engineered bacterial cells and various purification methods to recover active proteins from them have been recently reviewed¹⁰⁶. In some cases the formation of inclusion bodies may actually simplify their purification. The majority of contaminants are washed out after lysing the cells followed by solubilization of inclusion bodies in a denaturation and renaturation buffer^{107,108}. Figure 5 illustrates the extraction of a recombinant viral protein from inclusion bodies of *Escherichia coli*. In this case, urea was found to be the superior extracting agent to produce the protein in a state amenable for further purification and analysis. Detection of activity in situ in SDS-gels and renaturation of the protein may be enhanced by inclusion of a natural substrate as a template in the renaturation buffer^{68,109}. Alternatively, renaturation procedures may be unnecessary depending on the goal of the purification scheme.

physiological state of the cells must be standardized to obtain maximum enzyme synthesis.

4 Purification of Proteins in the Denatured State

4.1 Protein Native State

The amino acid sequence of a protein contains the information necessary for its folding into a biologically active three-dimensional form called the native state. However, in some cases it may be advantageous to purify proteins under denaturing conditions whereby conformation is abolished to maximize the resolution between the protein of interest and contaminants. Purification of proteins in denatured states followed by renaturation to a properly folded three-dimensional conformation has now been achieved for a number of proteins⁹¹.

4.2 Denaturation-Renaturation

The native state is usually the thermodynamically most stable form of a protein although it usually interconverts among a variety of native-like enzymatically active specific three-dimensional globular conformations. Many agents (organic solvents, heat, detergents, chaotropic salts, pressure) destabilize the native state of a protein resulting in the protein's loss of biological activity due to a change in conformation. However, these biologically inactive proteins can be renatured to their native state by slow removal of the denaturant⁶⁸. Slow dialysis in physiological buffer allows the protein to "shuffle" its way back to the native state⁶⁹. Some protein folding steps are quite slow and require several days to reach completion. Rates of dilution and denaturant concentration may dictate the pathway of refolding⁷⁰⁻⁷². The σ subunit of RNA polymerase, DNA topoisomerase⁷³, 1,25-dihydroxyvitamin D3 receptor⁷⁴, the α subunit of torpedo California acetylcholine receptor⁷⁵, estrogen receptor⁷⁶, chymotrypsin⁷⁷, urease⁷⁸, fructosyltransferase⁷⁹ and lactamase⁸⁰ have been renatured to biologically active native states after elution from SDS-PAGE gels^{81,82}.

Hydrophobic column packings can denature certain proteins^{83,84}. Denaturation can be overcome by minimizing on-column dwell time, lowering the temperature and using mild denaturant solvents such as triethylammonium phosphate^{85,86}. Improved recovery of hydrophobic proteins has been obtained by including denaturants in the mobile phase of reversed phase columns⁸⁷. Use of urea, guanidine hydrochloride, SDS and organic solvents avoids skew elution patterns in size exclusion chromatography^{88,89}. Viral coat proteins of Sendai virus⁹⁰, subunits of α crystallin⁹¹ and tubulin⁹² have been purified by using denaturing solvents in combination with high-performance ion exchange chromatography.

Detergent extraction and purification of membrane proteins has been reviewed⁹³⁻⁹⁵. Detergents can prevent proteolysis during crude extract preparation. However, many commercial preparations of detergents (e.g. Brij, Tween, Lubrol, Triton) are

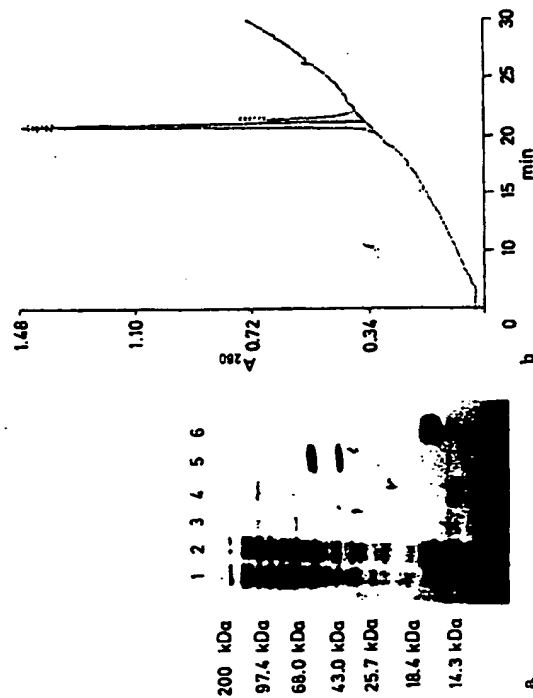


Fig. 5a, b. Purification of a recombinant protein from inclusion bodies. A segment of the genome of the human T-cell leukemia virus type I encoding the transmembrane envelope protein was cloned into plasmid pBR322 and used to transform *E. coli* strain HB101. Panel a illustrates a Coomassie blue stained SDS-PAGE analysis of a negative control extract of uninduced cells (lane 1), an extract of cells induced with heat (lane 2), the supernatant remaining after lysozyme extraction (lane 3), the inclusion body pellet following lysozyme treatment subsequently extracted with deoxycholate and Triton X-100 (lane 4), followed by octyl β -glucoside (lane 5), followed by 8 M urea (lane 6). The positions of molecular weight markers are indicated on the left in kDa. A major band at 16,000 Da was observed following urea extraction from which minor high molecular weight contaminants were removed by size exclusion chromatography on a Sephadex G-75 column. Panel b reveals that a single, prominent peak was observed when this preparation was analyzed by reversed phase HPLC indicative of its homogeneity. Courtesy of Dr. Chang-Chih Tai, Biotech Research Laboratories and Dr. Mark Connelly, DuPont.

For example, since many recombinant proteins are produced in denatured form, antibodies raised against denatured protein may be more effective than antiserum against the native state for screening a cDNA library for expression of eukaryotic proteins ¹¹⁰.

Proteins can be renatured by the slow dialysis or dilution method after complete denaturation to random coils in a solution of chaotropic salt. Concentrated urea solutions, which yield protein-reactive cyanate ions and guanidine salts are, therefore, preferable. Proteins purified by SDS-PAGE can be eluted into SDS-containing buffer by diffusion, precipitated with acetone and denatured ^{73,111}. Inclusion of detergents in the refolding buffer or pretreatment of proteins with detergents may counteract aggregation and thus facilitate protein renaturation ⁷³. Renaturation of proteins containing disulfides has also been achieved ¹¹²⁻¹¹⁴.

5 Preparation of Cell-Free Extracts

For preparing cell-free extracts, the methods of choice depend upon the state of the material. Animal organs (liver, kidney, etc.) and various plant tissues can be homogenized in a "Waring blender" with a buffer after freezing. Bacterial cells are broken either by freezing and thawing, grinding with alumina, glass beads or sand, sonication or use of a "French press". There are various types of mills commercially available to break microbial cells. The buffer should contain 20% glycerol or ethylene glycol to stabilize enzyme activity. Many successful purification schemes have relied upon the presence of several different protease inhibitors particularly during steps which denature proteins. For example, the following mixture has been recommended as a starting point ¹¹⁵: 1 μ M leupeptin (for serine and thiol proteases), 200 μ M phenylmethylsulfonyl fluoride (PMSF, for serine proteases), 1 μ M pepstatin (for acid proteases) and 100 μ M EDTA (for metalloproteases). Protease inhibitor solutions should be prepared fresh daily and added at each step of the purification scheme since many are unstable in aqueous solution (e.g. PMSF). Furthermore, many inhibitors are only effective against active enzymes and will not affect proteases that naturally exist as inactive complexes with endogenous inhibitors but which are activated upon dissociation. Polyvinyl pyrrolidone and nonionic polymeric absorbents such as amberlite XAD-4 (Sigma Chemical Co., St. Louis, MO) are added to prevent inactivation of proteins by phenols.

6 Bulk Precipitation and Phase Extraction

Once the protein has been solubilized, an early purification step often involves salt or alcohol mediated precipitation followed by resolubilization and desalting. The objective of this procedure is two-fold. The first is to reduce the volume of the starting material to a level amenable to subsequent purification steps. Second, gross impurities such as carbohydrates, lipids and nucleic acids are removed. Although the degree of purification achieved by precipitating techniques is modest, the contaminants that are depleted often interfere with later procedures ³⁵. Fractionation of protein mixtures by precipitation with organic solvents such as ethanol and acetone, ammonium sulfate and polyethylene glycol is often used. Ammonium sulfate is the preferred precipitant because of its high solubility (up to 4 M), low cost, and its stabilizing effect on the activity of most enzymes.

Protein precipitation by various agents has been reviewed ¹¹⁶. The charged polymer polyethylenimine (PEI) has been used in protein purification. PEI forms ionic complexes with and thus precipitates macromolecules containing acidic domains such as nucleic acids and proteins. Salt concentration, pH and the concentration of the material to be precipitated affect the degree of precipitation. Instead of using protamine sulfate or streptomycin sulfate, PEI would be used to remove nucleic acids and nucleoproteins from crude extracts followed by ammonium sulfate precipitation and chromatography. Usually clear supernatant fractions are obtained after formation of charge neutralization complexes and creation of cross bridges between complexes by PEI. The pKa value of the imino groups of PEI is 10 to 11 and fractionation is done at slightly alkaline pH.

Aqueous two-phase systems based on the behavior of hydrophilic polymers in solution provide an extraction technique for purification of enzymes on large-scale. Cell debris and the desired enzyme are partitioned into opposite phases independent of temperature. The selectivity of partition is further enhanced by covalent attachment of an affinity ligand for the desired enzyme to a phase-forming polymer and performing the extraction under conditions which allow ligand-enzyme complex formation. Polyethylene glycol (PEG)-salt systems which require high salt concentrations will dissociate affinity ligand-enzyme complexes. Affinity extraction of formate dehydrogenase from *Candida boidinii*¹¹⁷. A triazine dye was bound to PEG and the enzyme-ligand complex was extracted in the top phase. The complex was dissociated by the addition of salt expelling the ligand to the top phase and the enzyme to the bottom phase. Three-phase partitioning methods using ammonium sulfate and *t*-butanol for separating enzymes in mid-layer have been reported¹¹⁸. Besides purification of the desired enzyme, three-phase partitioning extracts apolar pigments into the *t*-butanol layer yielding a clean extract.

7 Conventional vs High-Performance Liquid Chromatography

7.1 The Principle of Liquid Chromatography

Chromatography is based upon the principle of differential partition of a specific component between two phases, the mobile phase and the stationary phase. The former serves to move the sample through the latter in a manner that optimizes the physical and chemical interactions of the sample with both phases. The mobile phase can exist in either the liquid or gaseous state while the stationary phase commonly occurs as a liquid or solid. Protein separations are normally performed in a combination referred to as liquid chromatography which employs a liquid mobile phase and a solid stationary phase packed into a tubular shaped column.

7.2 High-Performance Liquid Chromatography

The past ten years have witnessed a revolution in the development of new technologies directed towards the purification of proteins from complex biological fluids. Particularly important among these are high-performance liquid chromatographic methodologies. Compared to conventional liquid chromatography, HPLC offers the distinct advantages of superior resolution and speed of operation. This principle is portrayed in Fig. 6. Although traditional liquid chromatographic techniques are easy to use and operate at low financial cost, they are conducted on columns containing chromatographic media composed of carbohydrate or synthetic polymer supports with particle sizes generally in the range of 50 to 150 μm . As a result of these relatively large and heterogeneous sizes, these media provide for only poor resolution of protein mixtures. Furthermore, their susceptibility to volume compression due to high linear flow velocities necessitates slow flow rates and correspondingly long

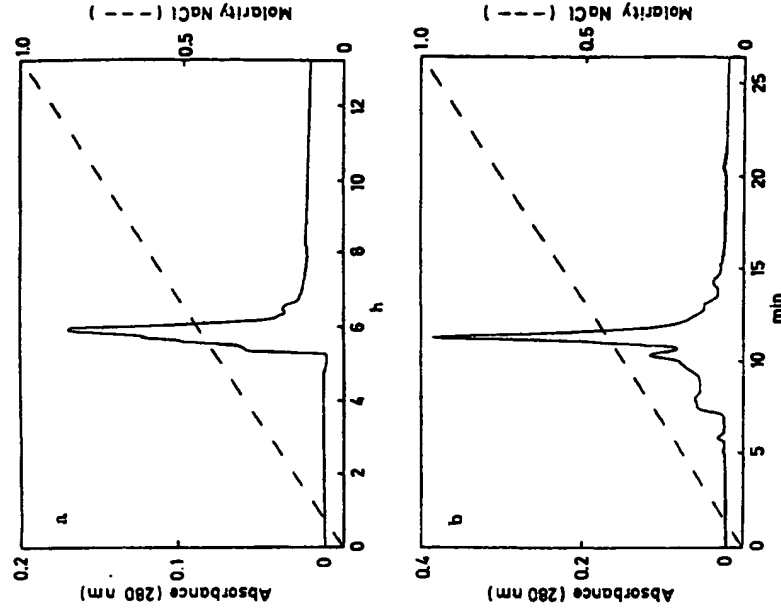


Fig. 6a, b. Comparison of conventional ion exchange chromatography with HPLC. A commercial preparation of ovalbumin was separated using either a standard, low pressure DEAE-cellulose column (1.5 x 15 cm; panel a) at a flow rate of 25 ml h^{-1} or a DEAE SPW HPLC column (Waters; 2.15 x 15 cm; panel b) at a flow rate of 4 ml min^{-1} . A linear gradient from 0 to 1 M NaCl in 10 mM sodium phosphate buffer, pH 7.2 was used for elution. Note the increased peak resolution and reduced retention time produced with the HPLC column.

separation times. Normally this requires that such separations be performed at reduced temperature in order to preserve the protein of interest from degradative physical, chemical and/or enzymatic effects.

HPLC, introduced in the late 1970's, employs silica- or polymer-based supports to which are attached various functional groups to overcome the limitations of conventional liquid chromatography¹¹⁹⁻¹²¹. These supports are available in a wide array of particle sizes that can be manufactured with a high degree of uniformity. As a result, the resolution of protein mixtures by HPLC far surpasses that even remotely possible by traditional chromatography. Silica-based supports also possess the capability to withstand high linear flow velocities without significant compression effects allowing separations to be performed on a time-scale of minutes rather than

hours. As illustrated in Fig. 6, a typical ion exchange chromatographic separation by HPLC can utilize flow rates that are 10 to 15 times greater than those possible by conventional chromatography. Such separations may usually be performed at ambient temperatures without substantial degradative losses. Two additional advantages are provided by silica-based supports used in HPLC. First, the ability to modify the silica surface with a variety of chemistries provides the protein chemist with a large array of separation modes. Second, these particles are available in a wide range of pore sizes. Those with small diameter pores (100 Å or less) provide for large surface areas to allow increased interactions between the stationary and mobile phases. These are often used to separate small proteins and peptides by chromatographic techniques that utilize bonding interactions with the stationary phase, for example reversed phase and ion exchange HPLC. On the contrary, particles with large pore sizes (300 to 1,000 Å) allow increased accessibility for large proteins which makes them ideal for size exclusion HPLC.

The development of column packings with high performance, high resolution, speed, stability and rigidity has made it easier to isolate and purify proteins. Packings based on Sepharose and Toyopearl polymeric resins provide high flow rates and good recovery of proteins while preserving biological activity. Furthermore, like the silica-based resins employed in HPLC, their versatility allows them to accomplish size exclusion, ion exchange, chromatofocusing, reversed phase, hydrophobic interaction and affinity chromatography. Fast protein liquid chromatography (FPLC), developed by Pharmacia LKB Biotechnology, is such a chromatographic system. Utilizing matrices based upon Sepharose resins, it generally employs operating pressures between those of conventional and HPLC chromatographies. FPLC is therefore capable of combining the benefits of high resolution and speed characteristic of HPLC with biocompatibility, long column life and ease of scale up. Moreover, the modular design of the FPLC system allows the protein biochemist to assemble the specific configuration necessary for his or her needs. Since it is a total chromatographic system, all components from pumps to detector, recorder and fraction collector are designed into a single unit. Consequently, FPLC has gained world-wide popularity as for the purification of macromolecules, particularly peptides and proteins.

7.3 Mathematical Descriptions

A variety of mathematical formulas have been developed to describe the resolving characteristics of a particular chromatography system and these have been reviewed (23). The most popular are those describing the concept of theoretical plates (N), which is basically a measure of the band or peak width around the center of a Gaussian elution peak. Thus,

$$N = (V/\sigma)^2 \quad (1)$$

where V is retention volume, time or distance and σ is a measure of peak variance. Sigma (σ) is correctly described algebraically in terms of classical statistics. More commonly, however, peak width is determined by any of a number of simpler techniques such as extrapolation of inflection point tangents to baseline or measurement

at 4.4% of peak height. In either case, the larger the number of theoretical plates, the narrower the peak spreading that occurs. Column efficiency refers to the lack of band spreading as measured by the absolute number of theoretical plates for a given system or the height equivalent to a theoretical plate (HETP). If L is column length then

$$\text{HETP} = L/N \quad (2)$$

Resolution (R) is a measure of the separation of one component from another and is defined mathematically as the difference in elution volumes for two peaks divided by their average widths. The capacity factor (k') measures the ratio of the total amount of component on the stationary phase to the total amount in the mobile phase. The distribution coefficient (K) refers to the same ratio using units of concentration. Both terms reveal where components elute relative to the void volume (V_0), the elution volume of an unretained substance, and are unaffected by flow rate or column dimensions. The selectivity factor (α) is equal to the ratio of the k' values of two components and is a measure of how efficiently the mobile and stationary phases compete for a sample. The practical value of these terms lies in their ability to compare different chromatographic separations. For example, traditional low-pressure columns commonly possess HETP values of 100 to 300 theoretical plates per meter whereas a modern reversed phase HPLC column may exceed 100,000 plates per meter.

8 Descriptions of Chromatographic Techniques

In view of the dominance that HPLC technology has had over the field of macromolecular purification during the past decade, this section will be primarily devoted to recent developments in this area. A variety of HPLC methodologies exist that can

Table 1. Techniques of column chromatography

Separation mode	Characteristics
Size exclusion chromatography	Size separation and desalting of proteins, oligosaccharides and nucleic acids. Fractionation range from 100 to 50,000 Da.
Ion exchange chromatography	Purification of proteins and enzymes by charge. High resolution and speed with very high capacity. Compatible with preserving bio-activity.
Hydroxylapatite chromatography	High capacity with low degree of nonspecific binding. Unique separation parameter not primarily dependent on size, charge or hydrophobicity allowing resolution of complex mixtures when other methods fail.
Hydrophobic interaction chromatography	Purification of proteins by hydrophobic nature. Best suited when sample is of high ionic strength (e.g. after salt precipitation).
Reversed phase chromatography	Purification of peptides, fatty acids and small molecules by liquid-liquid partition.
Affinity chromatography	Very specific purification of proteins utilizing their biological affinity to ligands bound on solid support.

be applied to a given scheme to purify an individual protein from a complex mixture of components. Different techniques are based upon different physical or biological properties exhibited by polypeptides and proteins such as their hydrodynamic volume, ionic character, hydrophobicity (surface and interior) and ligand affinity. The following section will highlight the theoretical and practical considerations relating to these methods, viz. size exclusion, ion exchange, hydrophobic interaction, reversed phase and affinity chromatography. Table 1 lists the types of column chromatography techniques. Table 2 presents the commercial suppliers of conventional chromatography resins and HPLC columns.

Table 2. Commercial suppliers of chromatography resins and columns

Supplier	Conventional resins					HPLC columns				
	SEC	IEC	HAC	HIC	AC	SEC	IEC	HAC	RPC	HIC AC
Baker		x		x	x		x		x	x
BioRad						x	x		x	x
Brownlee (ABI)	x	x	x		x	x	x	x	x	x
Calbiochem										
DuPont			x		x	x	x		x	x
EM Science	x					x	x	x	x	
Hamilton						x	x			
IBF Biotechnics	x				x					
ISCO						x	x		x	x
Pharmacia LKB	x	x		x	x	x	x		x	
Phenomenex	x	x		x	x	x	x		x	x
Pierce		x				x	x		x	x
Rainin						x	x		x	
Separations Group										
Sigma	x	x	x		x					
Supelco						x	x	x	x	x
Synchrom						x	x		x	x
ToyoHaas						x	x		x	x
Waters (Millipore)	x	x		x	x	x	x		x	x
Whatman						x	x		x	

SEC: Size exclusion chromatography;
 IEC: Ion exchange chromatography;
 HAC: Hydroxylapatite chromatography;
 RPC: Reversed phase chromatography;
 HIC: Hydrophobic interaction chromatography;
 AC: Affinity chromatography

8.1 Size Exclusion Chromatography

Historically, size exclusion (gel filtration, gel permeation) chromatography was the first of the chromatographic methods to be adapted to HPLC^{119,121}. Macromolecules in the mobile phase differentially penetrate the pores of the stationary phase particles according to their hydrodynamic volume such that the larger components

are excluded from the interior of the particles whereas the smaller molecules are accessible to this volume. Thus, under ideal conditions, the order of elution can generally be predicted by the size of the protein since a linear relationship exists between its elution volume and the logarithmic value of its molecular weight. Operationally, however, some degree of interaction between the protein and size exclusion support cannot be avoided although these effects can be minimized by addition of modifiers such as salts¹²⁴ or detergents¹²⁵ to the mobile phase. A recent study on the chromatographic behavior of polypeptides and proteins in the range between 200 and 200,000 Da by size exclusion chromatography in aqueous solution concluded that the effects which determine their relative separations are dependent upon their molecular weight¹²⁶. Thus, proteins with molecular weights greater than 3,000 Da were resolved predominantly based upon their hydrodynamic radii. In contrast, for polypeptides less than 900 Da separations were mainly influenced by their ionic character and hydrophobicity. Bennett et al.¹²⁶ observed that these ionic and polar interactions could be minimized by utilizing a mobile phase consisting of 40% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA). This system allowed accurate estimation of molecular weights of small proteins in the range of 500 to 15,000 Da which was attributed to the hydrophobic properties of acetonitrile and the ion-pairing properties of TFA inhibiting nonpolar and polar interactions with the support. Although the application of high-performance technology to size exclusion chromatography has greatly enhanced its resolving power, this technique still remains one of the least effective means of separating protein mixtures in the armamentarium of HPLC. Proteins with a difference in molecular weight of 10% or less often cannot be separated. By comparison, peptides with a single amino acid substitution can be resolved by reversed phase HPLC¹⁴.

A common practice used to increase the theoretical plate number (N) of size exclusion separations is to connect in series two or more columns containing the same pore size support. Similarly, since the effective molecular weight fractionation range is determined by the pore size rather than the column length, connection of two columns with differing pore size supports in tandem will increase not only N but also the fractionation range (Fig. 7). Most commercially available columns will separate proteins over about 1.5 to 2.5 orders of magnitude in size. However, by connection of two columns containing supports with pore sizes differing by a factor of 10, the effective separation range can be doubled¹²⁷. Furthermore, this method of bimodal coupling produces more linear calibration curves than using a single column containing a mixture of particles with different pore sizes. Operationally, the columns should be connected in order of decreasing pore size and the individual calibration curves of each column should minimally overlap to achieve the maximum combined fractionation range.

Size exclusion chromatography is an excellent technique to employ early in a purification scheme, ideally as the first or second step, since it results in sample dilution. Proteolysis due to chemical or enzymatic effects can therefore be minimized at an early stage in purification with a resulting higher yield in the final product. A second clear advantage of size exclusion chromatography is the ability to select from a wide variety of mobile phases. Generally, mobile phases are buffered, low salt aqueous solutions (e.g., 0.25 M sodium acetate containing 0.25 M sodium chloride, pH 5.5) used to maintain native structure. However, extreme conditions of pH as

8.2 Ion Exchange Chromatography

Ion exchange chromatography of proteins involves the interaction of charged functional groups in the sample with ionic functional groups of opposite charge on the adsorbent surface. Two types of electrostatic interactions can be distinguished. Anion exchange chromatography is mediated by the interaction of negatively charged amino acid side chains (e.g., aspartic acid and glutamic acid) with positively charged supports. Proteins with a net negative charge, i.e. in an aqueous solution with a pH above their isoelectric point (pI), will bind to these supports. Cation exchangers are prepared by polymerization of negatively charged groups to silica for interaction with positively charged residues (e.g., lysine and arginine). Proteins in a solution with a pH below their pI will interact with these adsorbents. Further differentiation among ion exchange resins can be made according to their ionic capacity, commonly referred to as "weak" and "strong" although these terms do not refer to the strength of binding between the sample and matrix. Thus, a weak anion exchange resin would be provided by the diethylaminoethyl (DEAE) group whereas the quaternary aminoethyl (QAE) group is a strong anion exchanger. Examples of weak and strong cation exchangers are the carboxymethyl (CM) and sulfoxypropyl (SP) groups, respectively. Phosphocellulose cation exchange chromatography utilizes the phosphoryl group which is of intermediate strength compared to CM and SP modified supports. Cellulose phosphate resins have also been prepared to provide bifunctional cation exchangers containing both fully ionized and weak cation groups. The advantage offered by this adsorbent is that it offers three different mechanisms of protein binding and elution.^{134a} First, it may be used as a normal cation exchange resin. Second, it may be used as an adsorbent to purify proteins with a specific affinity for phosphate groups.¹³⁵ Finally, the substrate elution technique allows one to bind an enzyme to the resin and elute it with a protein substrate or inhibitor.¹³⁶

The binding energy between the sample and adsorbent is dependent upon the solvent pH, ionic strength and selectivity of the counter-ion. Thus, bound components can be differentially eluted by alterations in any of these three parameters. Common practice has relied on constant conditions of pH and counter-ions with a gradient of increasing salt concentration. These conditions are normally compatible with maintaining native biological or enzymatic activity. However, if such activity is adversely affected by extremes in ionic strength, gradients of pH or selection of a different salt counter-ion may be employed.¹³⁷⁻¹⁴¹ Utilization of a different salt counter-ion also affects the differential resolution of components in a complex mixture. Thus, protein separation on an ion exchange HPLC column can be facilitated by either optimizing the buffer conditions for a given chromatographic run or by rechromatography of the protein of interest on the same column in the presence of an alternative salt counter-ion. Proteins that co-elute under the conditions of one buffer system may be easily resolved by another system. For example, whereas reduced and oxidized forms of cytochrome C could not be separated on a weak cation exchange column using gradients of NaCl or $(\text{NH}_4)_2\text{SO}_4$, the same column produced a discernable resolution using NaOAc and baseline separation with KII_3PO_4 as the counter-ion.^{141a}

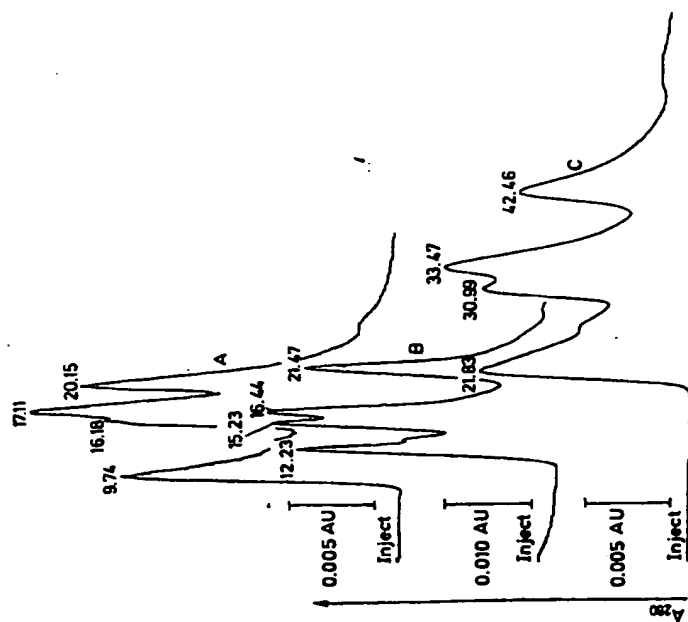


Fig. 7. Connection of two HPLC size exclusion columns in series. A standard protein mixture consisting of blue dextran (2,000,000 Da), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da) and chymotrypsinogen (25,000 Da) was resolved on a Protein Pak 300 SW column (A; 0.75 x 30 cm; fractionation range 10,000–400,000 Da; Waters), a Protein Pak 125 column (B; 0.75 x 30 cm; fractionation range 2,000–80,000 Da; Waters) or the two columns connected in series (C). The flow rate was 0.5 ml min⁻¹ using 10 mM Tris, pH 7.5 containing 0.5 M NaCl. Retention times in min of each protein are given above the peaks.

well as the presence of organic solvents, high concentrations of salts and/or denaturing agents such as urea or guanidine may be employed to minimize aggregation effects while maintaining sample solubility.^{7,138,139} Under these conditions, protein activity may not be recovered. Recent widespread use of both ionic and non-ionic detergents has, in many cases, allowed proteins with unusual solubility properties to be isolated. Particularly illustrative of this is the purification of integral plasma membrane proteins¹³⁰⁻¹³³. Ionic detergents (e.g. SDS) are not as well suited as nonionic ones for extraction of membrane proteins because they are known to denature proteins. Other notable examples of the utility of size exclusion chromatography over other HPLC techniques are the study of protein folding and stability¹³⁰ and the dissociation constants of oligomeric polypeptides.¹³⁴

chromatofocusing, as its name implies, also relies on a focusing effect that is developed by cycles of sample adsorption to and desorption from the solid support. This principle is illustrated in Fig. 9. The sample is applied to the resin at high pH following with a buffer of lower pH containing various amphoteric components with a range of pKa values is used to titrate both the adsorbent and bound sample. The former

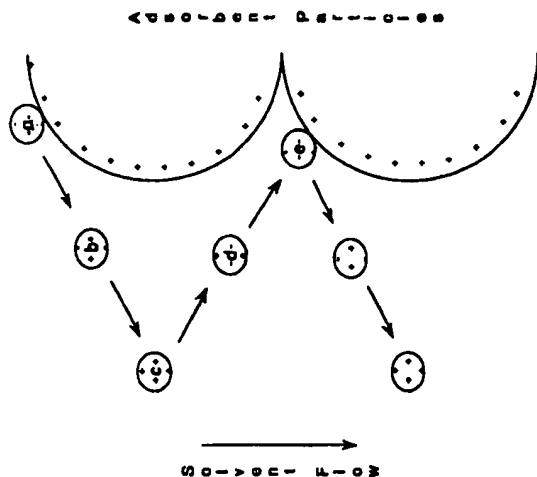


Fig. 9. The principle of chromatofocusing. The protein component with a net negative charge (a) is initially retained on the positively charged adsorbent. As the pH gradient is formed, the proteins at the rear of the sample zone are titrated first to neutrality (b) and then to a net positive charge (c) resulting in their repulsion from the adsorbent and subsequent migration into the front of the sample zone. Here the increase in pH results in their titration back to neutrality (d) and back to a net negative charge (e) leading to reabsorption to the matrix and their return to the rear of the sample zone, completing the chromatofocusing cycle.

results in a linear, descending pH gradient. As this pH gradient develops, the proteins at the rear of the sample zone are the first to be titrated to the same charge as the support and consequently desorbed from the support allowing them to migrate to the front of the sample zone. At this point they are titrated back to a charge opposite that of the adsorbent and reabsorb to it. Consecutive cycles of exchange between the front and rear of the sample zone results in a focusing of this zone. Proteins comprising a crude mixture will effectively be separated based upon their isoelectric point (pI) with those possessing pIs near the initial column pH eluting first and those with pIs different from the initial pH eluting later. Like ion exchange HPLC, a multitude of different proteins have been isolated by chromatofocusing.¹⁵³⁻¹⁵⁶

Generally, ion exchange HPLC produces poorer resolution than reversed phase HPLC although it is superior in recovering biological activity. However, exceptions have been noted, as in the case where ion exchange chromatography separated three polypeptides of diphtheria toxin A that differed only at their carboxyl termini and could not be resolved by reversed phase HPLC.³⁷ Resolution of proteins that differ by only a single charge has also been reported.¹⁴² Many other examples of protein separations by HPLC on ion exchange resins can be cited. These include enzymes^{143,144}, peptide hormones¹⁴⁵, hormone receptors¹⁴⁶, serum proteins^{147,148} and structural proteins^{149,150}. These citations should not be considered as inclusive but rather as a general guide to illustrate the widespread application of ion exchange chromatography to protein isolations. Indeed, a recent compilation of proteins separated by ion exchange FPLC lists 127 different polypeptides from over 175 literature references¹⁵¹. Prominent among this list are mouse monoclonal antibodies. Figure 8 illustrates the large scale isolation of an IgG1 monoclonal antibody by anion exchange FPLC. This example is particularly noteworthy in light of the fact that this isotype cannot be readily purified by conventional protein A immunoaffinity chromatography (see below).

Chromatofocusing is a relatively new technique that, like ion exchange chromatography, separates macromolecules on the basis of their net ionic charge¹⁵². However,

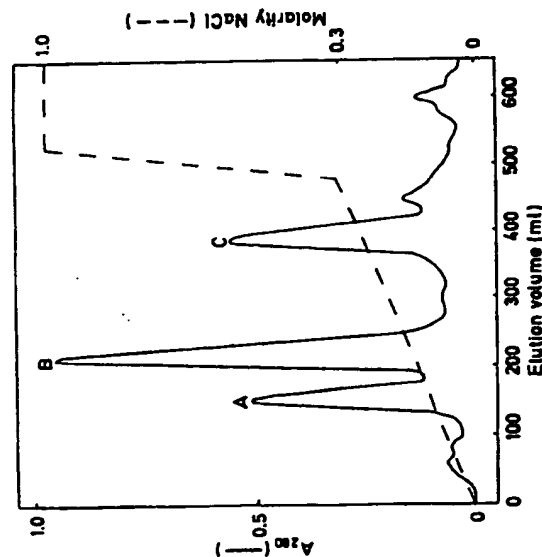


Fig. 8. Scale-up purification of a mouse monoclonal IgG1 antibody by ion exchange FPLC. Five milliliters of mouse ascites was loaded onto a Mono Q anion exchange column (0.5 x 5 cm; Pharmacia LKB Biotechnology) and the proteins eluted in 50 mM Tris, pH 8.6 with a 0 to 1.0 M gradient of NaCl as indicated at 10 ml min⁻¹. The major peak (B) eluting between 200 and 250 ml was observed by SDS-PAGE to contain > 95% pure monoclonal antibody. Peaks A and C were identified as transferrin and albumin, respectively. Note: the isotype of this antibody precluded its isolation by protein A affinity chromatography. Courtesy of Dr. Steve Alexander, Biotech Research Laboratories.

8.3 Hydroxylapatite Chromatography

Hydroxylapatite is a crystallized derivative of calcium phosphate originally developed by Tiselius and coworkers¹⁵⁷⁾ for fractionation of complex protein mixtures. Proteins adsorb to it via ionic interactions and can subsequently be quantitatively eluted in a high ionic strength buffer. Since phosphate buffers are generally compatible with protein structure and function, increasing gradients of phosphate concentrations (e.g. 0.01 to 0.50 M) are commonly used to desorb bound components. It has been suggested that the actual mechanism of interaction between proteins and hydroxylapatite involves carbonyl and phosphate groups on the surface of native proteins¹⁵⁸⁾. Urea-induced protein denaturation was observed to decrease the binding to hydroxylapatite presumably as a consequence of a reduction in the number of these ionic groups on the surface of proteins in a random coil configuration¹⁵⁹⁾. However, others have observed that SDS, which also produces random coil denaturation, can be used in conjunction with hydroxylapatite chromatography for the separation of polypeptides¹⁶⁰⁾. Indeed, an advantageous feature of this method is that protein elution is independent of molecular weight thus making it a powerful companion to use following separation by size exclusion chromatography or polyacrylamide gel electrophoresis in the presence of SDS.

Although hydroxylapatite chromatography is based upon ionic adsorption, it provides a unique fractionation method with properties different from those of ion exchange chromatography. This fact, together with its high binding capacity, makes it a popular method of protein purification. Brooks¹⁶¹⁾ has reviewed the applications of hydroxylapatite chromatography to protein isolations including those of enzymes, chromatin proteins, glycoproteins, lipoproteins, phosphoproteins and lectins. Although this list is impressive, many practical problems have been encountered in handling this material, particularly those related to the inherent fragility of the crystals. However, careful attention to particle size allows high chromatographic resolution of proteins on this medium. Recently, hydroxylapatite HPLC columns have become commercially available and proven useful for the purification of peptides and proteins¹⁶²⁾.

8.4 Reversed Phase Chromatography

In normal phase chromatography, the solvent is less polar than the adsorbent. Reversed phase chromatography uses a solvent that is more polar than the adsorbent. Silica is by nature very polar allowing normal phase chromatography to be performed directly on these particles. More commonly, however, silica is bonded with a polar functional group such as the cyano or amino groups and normal phase chromatography performed with a mobile phase such as hexane, methylene chloride, chloroform or ether. Silica, bonded with nonpolar functional groups such as alkyl or aromatic phenyl groups, is employed in reversed phase chromatography. In this case, the nonpolar sample constituents bind to the support pre-equilibrated in a polar solvent such as water or an aqueous buffer and are eluted with a mobile phase containing acetonitrile, methanol, isopropanol or tetrahydrofuran. Such elutions are performed

utilizing either isocratic conditions (i.e., constant mobile phase composition) or gradients of increasing organic solvent. Stationary phases containing bonded alkyl groups (butyl, C-4; octyl, C-8; octadecyl, C-18) are the most popular reversed phase supports. Although there is minimal variation in the retention times of proteins on these different supports¹⁶³⁾, better recoveries have been observed using the shorter carbon chain groups¹⁶⁴⁾.

Reversed phase chromatography was initially applied to the separation and purification of small peptides since it was shown that even minor differences in their amino acid composition greatly affected their elution profile¹⁶⁵⁾. Furthermore, the relative content of hydrophobic amino acids can sometimes be used to predict their elution order. Conditions were developed that produced baseline separations of complex peptide mixtures, often by incorporating an ion-pairing agent (commonly dilute TFA) to elute components as one member of the ion-pair¹⁶⁶⁾. Reversed phase chromatography does, however, possess some handicaps particularly when attempting to isolate larger proteins. Prediction of elution order is often limited by secondary and tertiary structures that may prevent some hydrophobic amino acids from interacting with the support. Second, reproducibility of separations is often poor when comparing different commercial column sources or even comparing the same column from a single source¹⁶⁷⁾. Finally, native protein structure may be lost using standard organic solvents and acidic conditions of the mobile phase and hydrocarbon bonding in the stationary phase thus destroying biological activity or antigenic characteristics necessary for enzyme-based or immunological-based detection assays¹⁶⁸⁻¹⁷⁰⁾. The denaturative effects can be investigated on a small scale prior to applying a valuable sample to a reversed phase column by first preincubating it under conditions of the reversed phase chromatographic run. However, although denaturation may occur, post-column conditions of lyophilization or dialysis, in many cases, allow some degree of renaturation to occur. In fact, the list of proteins observed to undergo reversible denaturation following reversed phase HPLC is impressive. Selected examples of these include those of viral¹⁷¹⁾, bacterial¹⁷²⁾, plant¹⁷³⁾ and animal¹⁶⁹⁾ origin. Figure 10 illustrates the recovery of biologically active cytokines isolated on a reversed phase column. In some cases, the native and denatured forms of a protein can be resolved allowing the kinetics of denaturation to be investigated as demonstrated by a beautiful series of papers by Karger and coworkers^{163,167,172,174)}.

The major advantages that reversed phase chromatography has over other methods of HPLC are its high degree of resolving power and its versatility. Both properties lead to a generalized scheme often employed in HPLC methods development¹⁷⁵⁾. First, one identifies the particular chromatographic elution parameters of the protein of interest, for example by determining its retention time during a gradient of organic solvent. Once this is established, a variety of factors can be changed to produce a baseline separation of the protein such that it is free of contaminants that elute nearby. Changing columns from different vendors may dramatically alter retention times in spite of the fact that particle size, pore size and carbon coverings may be identical. Alternatively, solvent optimization using mixtures of acetonitrile/propanol or acetonitrile/methanol¹⁷⁶⁾ and chemical optimization by varying solvent pH¹⁷⁷⁾ or ion-pairing agents are possible. For example, Fett and coworkers¹⁷⁸⁾ observed that the purification of human angiogenin by reversed phase HPLC was dramatically improved by changing from a linear gradient of acetonitrile to a gradient of 2-pro-

8.5 Hydrophobic Interaction Chromatography

The tendency of organic solvents to denature proteins during reversed phase HPLC separations has been complemented by the development of hydrophobic interaction chromatography. This has been a particularly important development in the field of protein purification since this denaturative effect requires that protein characterization by reversed phase chromatography alone be dependent upon some knowledge of their molecular weights¹⁰⁰. As its name implies, hydrophobic interaction chromatography, like reversed phase chromatography, relies upon hydrophobic interactions between the protein sample and the adsorbent. However, unlike reversed phase supports, the bonded phases utilized in this method contain lower ligand densities and shorter ligand lengths (e.g. phenyl and butyl groups). As a result, surface hydrophobicity is reduced allowing bound proteins to be eluted under mild conditions, i.e. neutral pH and high ionic strength. Proteins initially bind to the support in the presence of high concentrations of salt (e.g., 2 to 4 M ammonium sulfate). The mechanism of binding depends upon the fact that many proteins, because of folding constraints caused by a variety of factors, such as disulfide bridging and glycosylation sites, contain hydrophobic areas on their surfaces. Under conditions which favor hydrophobic interactions, such as high salt concentrations found during a salting-out procedure, proteins will bind to nonpolar supports by way of these hydrophobic surface patches. During a gradient of decreasing salt concentration the bound proteins will then be differentially desorbed from the adsorbent according to their increasing hydrophobicities. Addition of an organic modifier such as methanol, ethanol or ethylene glycol to the eluting buffer increases its hydrophobicity and facilitates desorption off the column.

Although the underlying forces which determine the retention of proteins by hydrophobic interaction HPLC are recognized, understanding of all the effects which are involved is not complete. For example, it has been observed that protein retention on a hydrophobic interaction column is more sensitive to ligand length than is the case with reversed phase chromatography¹⁰¹. This was attributed to the hypothesis that in addition to nonpolar regions on the protein surface, sequestered hydrophobic residues in "pockets" near the surface are available to interact with the support ligand. The availability of these "pockets" depends upon the ability of the protein to undergo small conformational changes short of complete denaturation making it difficult to predict the elution order of different proteins in a mixture.

On a theoretical basis, proteins are likely to exhibit greater differences in their properties at their surfaces while in a native, globular form compared to a denatured form¹⁰². This accounts for why hydrophobic interaction chromatography is often reported to be equal to or in some cases superior to reversed phase chromatography in its ability to resolve protein mixtures⁹³. The list of proteins and polypeptides that have been successfully purified by hydrophobic interaction chromatography is equally impressive as that indicated above for reversed phase HPLC. However, two notable examples should be emphasized. Because of its less disruptive elution conditions than reversed phase chromatography, it is particularly effective in retaining enzymatic activity^{103,104} although enzymes that are inactivated by conditions of high salt concentrations are obvious exceptions¹⁰⁵. Second, hydrophobic interaction HPLC has been used to purify proteins that undergo ion-dependent

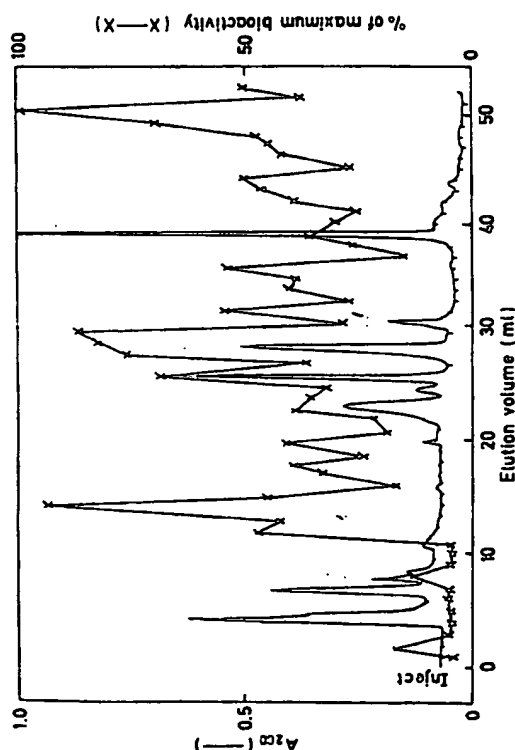


Fig. 10. Purification of biologically active cytokines by reversed phase HPLC. Conditioned medium from a Marek's disease virus transformed chicken T lymphocytic cell line was concentrated by lyophilization and resolved on an octadecyl (C-18) HPLC column developed with a linear 0 to 50% gradient of aqueous acetonitrile at pH 3.0. Individual fractions were concentrated by lyophilization, reconstituted in physiological buffer solution and assayed for bioactivity

panol/acetonitrile. Although this scheme is theoretically attractive, it is limited in its applicability by financial and time constraints. Testing a variety of different reversed phase HPLC columns from various suppliers can quickly develop into a very expensive undertaking with no guarantee of success. Furthermore, complete determination of the effects of solvent composition on resolution can be a time-consuming operation. In light of these restrictions, Berkowitz et al.¹⁷⁹ have proposed an integrated approach utilizing both conventional low pressure and high-performance liquid chromatographic techniques for protein purification. The first step of this scheme uses short (e.g. 5 cm) HPLC columns containing 3 to 5 μ m silica-based packings to optimize a particular purification on an analytical scale. Low financial cost, quick analysis time and reduced sample consumption are the advantages procured when using these shorter columns compared to the standard 4.6 x 250 mm HPLC columns containing 5 μ m particles. Once separation conditions have been optimized, inexpensive 40 μ m silica-based particles are employed in the second step on a preparative-scale using traditional, low pressure chromatography hardware. These media permit high linear flow velocities without compressibility problems inherent in softer, carbohydrate-based packings and are thus ideally suited for use during the initial stages of protein isolations. Finally, 3 to 15 μ m particles in standard-size HPLC columns are utilized in the third step. These provide high resolution separations required near the end of a purification scheme to remove contaminants that have copurified with the protein of interest due to similarities in their physical and chemical properties.

tertiary structural changes. α -Lactalbumin was isolated from milk whey by binding to a nonpolar support in the absence of calcium ions and eluting in an aqueous buffer containing Ca^{+2} ions¹⁸⁵. Other proteins which undergo the opposite effect, i.e., a decrease in surface hydrophobicity in the absence of Ca^{+2} ions, have been purified by first binding to the column in the presence of Ca^{+2} ions and eluting in the presence of chelating agents¹⁸⁶⁻¹⁸⁸. Thus, one can readily appreciate the potential applications that hydrophobic interaction chromatography offers to the protein chemistry laboratory.

8.6 Affinity Chromatography

Of all the chromatographic techniques available, affinity-based methodologies are the most versatile and in some respects the most powerful. Similar to ion exchange, reversed phase and hydrophobic interaction chromatography, affinity chromatography is founded on the principle of constituents being adsorbed to and subsequently eluted from a solid support. The unique property that this technique offers is that the initial binding of the constituent is to a conformationally complementary surface which confers a level of specificity unparalleled by other methods. Affinity elution, like elution of the enzyme by the substrate, can leave all impurities behind and elute the enzyme alone off the column.

Specific interaction between a ligand and macromolecule is a universal feature of biological systems. In the realm of protein chemistry, these interactions are found between an enzyme and its substrate, inhibitor and cofactor, an antibody and antigen, a lectin and glycoprotein, as well as a hormone or vitamin and its receptor. In general, two types of affinity-based separations can be distinguished. Individualized interactions occur between one specific ligand and one protein as, for example, the reaction of a monoclonal antibody and its immunogen. In addition to these specific types of bindings between single substances, group specific adsorbents have been prepared that possess affinity for a certain class of related substances. These allow several substances with affinities for a given ligand to be purified without the need to prepare a different adsorbent for each. Thus, an immobilized lectin can be used to isolate a variety of different glycoproteins that possess similar carbohydrate moieties on different polypeptide backbones.

Although certain biological interactions had been exploited prior to the late 1960's in attempts to purify specific proteins, affinity chromatographic techniques did not become popular until after this time. Axen et al.¹⁸⁹ reported that carbohydrate-based gels could be modified with cyanogen bromide to provide a matrix for covalent coupling to primary amino groups of proteins. Since this initial observation, the list of different types of matrices and coupling agents that can be utilized to prepare an insoluble ligand support has grown tremendously. However, unlike the other methods of chromatography that have been successfully adapted to HPLC, affinity chromatography is only recently finding application in this area. This is in large part due to the fact that the advantage of high resolution offered by HPLC-based separations may not be needed if affinity chromatography in a traditional, low pressure system is by itself able to achieve purification to homogeneity in a single, high-yield step. For example, Whiting and Lindstrom¹⁹⁰ reported a 21,000-fold purification of a nico-

tinic acetylcholine receptor protein by a single-step immunoaffinity chromatographic procedure. Contaminants that are present following affinity chromatography are generally few and in such low concentration that if the sample is not already sufficiently pure, only one additional step (e.g. HPLC-based) may be necessary. Robb and Kutry¹⁹¹ purified the human interleukin 2 receptor α chain by a two-step protocol utilizing immunoaffinity chromatography followed by reversed phase HPLC to remove a small percentage of remaining contaminants. Another limitation that has hindered the transition of affinity chromatography from conventional to HPLC chromatography is that silica-based supports may be incompatible with elution conditions commonly employed to desorb proteins. Silica particles are solubilized at high pH conditions⁹ thus preventing their use in those cases where alkaline buffers have been found to be most effective in eluting proteins from immunoaffinity columns^{192,193}. Figure 11 documents the immunoaffinity purification of an enzymatically active protein using 0.2 M NH_4OH , pH 11.0 for desorption.

The affinity HPLC column development that has occurred has been limited to analytical and small-scale preparative applications. Recently, however, the commercial availability of various preactivated HPLC affinity supports now makes this technique practical. Indeed, it does offer one distinct advantage unavailable with conventional, low-pressure affinity chromatography, i.e. its ability to ensure rapid neutralization of acidic or basic eluents thereby contributing to improved recovery of desorbed components¹⁹⁴. Future developments in HPLC affinity chromatography in the industrial setting are particularly promising. In this regard, commercial application of HPLC affinity chromatography should prove superior to other separation techniques in its ability to separate biologically active proteins generated by recombinant DNA technology. Since eukaryotic proteins synthesized in bacteria normally must undergo correct folding and disulfide bridging to form fully active species and this process is often only fractionally efficient, HPLC affinity chromatographic methods utilizing active site ligands that only bind to target proteins containing a properly folded catalytic site can be used to separate active forms of a protein from its inactive isomers^{195,196}.

From the above discussion, it can readily be appreciated why affinity chromatography is presently more popular in the basic research laboratory rather than industrial laboratory. A comprehensive publication describing the theoretical and practical aspects of this method compiled over 200 references¹⁹⁷. A few selected examples of these include the following: (1) purification of proteases such as elastase on a synthetic peptide substrate support¹⁹⁸ as well as esterase B¹⁹⁹ and cathepsin D²⁰⁰ on solid supports of their respective competitive inhibitor, (2) isolation of DNA-binding proteins²⁰¹ and enzymes^{202,203} on immobilized DNA, (3) use of carbohydrate supports for the purification of lectins^{204,205} and glycosidases²⁰⁶, (4) isolation of hormone-binding proteins and receptors utilizing affinity supports containing the specific hormone^{207,208} or its selective antagonist²⁰⁹, (5) purification of metalloproteins by metal ion chelate affinity chromatography^{210,211} and (6) probing the surface topography of electron donor residues (cysteine, histidine, tryptophan) of enzymes by metal ion affinity chromatography²¹².

Rather than review all realized and potential applications of affinity chromatography to protein purification, the remainder of this section will briefly emphasize two selected topics. The first is the use of antisera against synthetic peptides as solid

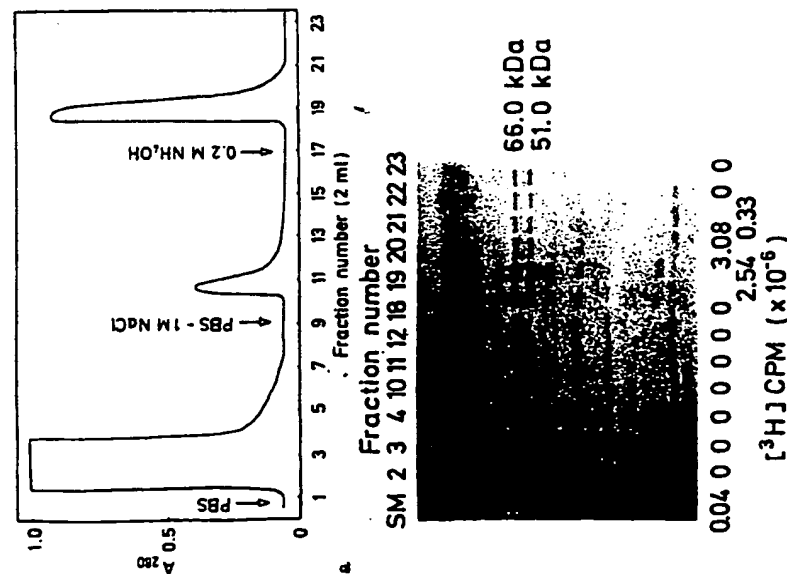


Fig. 11 a, b. Enzyme purification by immunoaffinity chromatography. A monoclonal antibody specific for the DNA-dependent RNA polymerase of human immunodeficiency virus type 1 (HIV-1) was coupled to CNBr activated Sepharose 4B, packed into a conventional low pressure column (2.5 x 2.0 cm) and used for the affinity purification of enzymatically active protein from a detergent extract of human lymphocytes infected with the virus. (a) illustrates the elution profile of unbound protein with phosphate buffered saline (PBS), pH 7.2, nonspecifically bound protein with PBS containing 1 M NaCl and specifically bound protein with 0.2 M NH₄OH, pH 11.0 (b) illustrates analysis of the starting material (SM) and selected elution fractions by immunoblotting with an anti-HIV-1 specific antiserum. The immunoblotting procedure involves separation of proteins by SDS-PAGE, electrophoretic transfer of the proteins to nitrocellulose and visualization by reaction with an antibody. Specifically adsorbed enzyme was eluted beginning at fraction 19 and appeared as two bands of 51.0 and 66.0 kDa, the smaller of which arises as a result of proteolytic cleavage of the C-terminus of the larger species. Enzyme activity, measured by the incorporation of [³H]thymidine into trichloroacetic acid insoluble RNA, is indicated below the Fig. and precisely correlates with the elution of antibody reactive protein. Courtesy of Dr. Robert Kilkuakle, Biotech Research Laboratories

supports for the purification of proteins from which the amino acid sequence of the peptide was deduced. The advent of recombinant DNA technology has resulted in the isolation and sequence analysis of a large number of genes for which either no known protein products exist or if they do exist, the proteins have not been purified. In many cases, these proteins can be identified and purified by affinity chromatography using antipeptide antibodies coupled to a solid support to adsorb the native protein followed by elution with the soluble peptide under mild conditions ²¹⁹. An additional step often involves a preliminary purification of the antipeptide antibodies on a peptide immunoabsorbent. These can then be eluted under acidic conditions ²¹⁴ or more gently with the free peptide ²¹⁵, the peptide removed by dialysis and the purified antibodies subsequently coupled to agarose to prepare an antipeptide affinity column for purification of the native protein. This method is fast and has been observed to produce a 2,500-fold purification in a single step ²¹⁶. Furthermore, it is a theoretically and technically feasible process to perform with the most difficult step being the selection of the appropriate amino acid sequence of the protein that will produce antibodies that react with the native protein. Several papers have summarized the important physicochemical properties of amino acids that should be considered when selecting a peptide sequence for immunization ²¹⁷⁻²¹⁹. The potential application of antipeptide immunoabsorbents to protein purification is enormous particularly in those cases where only small amounts of the protein exist.

The second area in which affinity chromatography has produced a tremendous impact is the purification of antibodies, particularly monoclonal antibodies, by use of *Staphylococcus aureus* protein A and more recently group G *Streptococcus* protein G. Both proteins bind to immunoglobulins, the difference between them being the species and isotype specificity of this interaction ^{220,221}. Protein A binds to human, rabbit, mouse and guinea pig immunoglobulins while protein G binds to all these as well as those from the goat, sheep, cow and horse. The latter also interacts with human IgG3 and mouse IgG1, two isotypes that possess very weak affinities for protein A. The affinities which both proteins A and G possess for the isotypes to which they do bind are very high. Therefore, the conditions necessary to dissociate these complexes are relatively harsh, for example buffers of low or high pH which may be strong enough to partially denature the immunoglobulin. A recent analysis of the effects of various physical treatments on several different monoclonal antibodies revealed that a 30 min incubation at either pH 3.0 or 10.0 resulted in a 25% to 65% reduction in antibody titer ²²². As an alternative method, the silica-based, mixed-mode antibody exchanger (AEx) liquid chromatography ion exchange column ²²³ has been shown to produce a single-step purification of monoclonal antibodies under mild conditions and is particularly useful for those isotypes that bind weakly to protein A including murine IgG1 ²²⁴ and IgM ²²⁵. The specificities of both proteins A and G make them ideally suited to purify monoclonal antibodies from either tissue culture supernatants or ascites fluids. Both bind to the Fc portion of immunoglobulins and thus they are useful as immunoabsorbents not only for purification of immune complexes without disruption of the antigen-antibody interaction ²²⁶ but also preparation of derivatives and fragments of IgG ²²⁷. In the former case, protein A immunoabsorbents have been utilized to isolate immune complexes between monoclonal antibodies and their corresponding radiolabeled antigens. Once dissociated, the antigens can be isolated in sufficient purity for radiochemical structural studies

amounts of sample and determining the maximum load that can be accommodated without surpassing the binding capacity of the column. Finally, the separation is directly transferred to a large preparative-scale column containing preparative-size particles.

The chromatographic elution conditions required for the process-scale separation are calculated based upon the respective dimensions of the analytical and preparative columns. For example, determination of the preparative flow rate (F_{Rp}) is made with the formula

$$F_{Rp} = [(Dp^2/Da^3) \times F_{Ra}] \quad (3)$$

where Dp is the diameter of the preparative column, Da is the diameter of the analytical column and F_{Ra} is the flow rate of the analytical column. Both F_{Rp} and F_{Ra} are related to their respective linear velocities (LV) which are experimentally determined with the equation

$$LV = L/Rt \quad (4)$$

where L is the column length and Rt is the retention time of an unretained peak such as acetone. From a plot of F_{Ra} vs. LVa and F_{Rp} vs. LVp , the actual value of F_{Rp} that equates LVp and LVa at the optimal value of F_{Ra} can then be deduced. Similarly, the scale-up factor (SF) used to determine the maximum mass load that can be accommodated when changing from an analytical to preparative-scale column is determined as

$$SF = (Dp^2 Lp)/(Da^2 La) \quad (5)$$

where Lp and La are the lengths of the preparative and analytical columns, respectively. A small-scale separation of 10 mg of protein on a 0.8×10 cm column can therefore be increased by a factor of 152 to 1.5 g on a 5.7×30 cm column.

10 Descriptions of Electrophoretic Techniques

Separation of proteins and polypeptides by electrophoresis has been the standard method of following a purification protocol and analyzing the homogeneity of the final product. However, the great resolving power exhibited by some of the more recently introduced modifications has also led to the development of preparative-scale electrophoretic techniques. Generally, proteins are electrophoresed through a support matrix, commonly paper, cellulose acetate, starch, agarose or polyacrylamide gel. Their rate of migration depends on the strength of the electrical field, the net charge, size and shape of the molecules as well as the ionic strength, viscosity and temperature of the matrix. Zonal electrophoretic methods utilize a buffer with a pH above the pI of the proteins in the sample and separations are based solely on net charge. Since agarose and polyacrylamide gels provide a porous matrix, these media will separate by charge and molecular weight. Agarose, a highly purified polysaccharide derived

by tryptic peptide mapping²²⁸⁾ or amino acid, sequence analysis²²⁹⁾. In these examples, since the objective was a primary structural study of the purified proteins, strong dissociating conditions could be used to separate the antigen from the immuno-adsorbent. However, in cases where conformation or biological activity must be preserved, milder elution conditions must be utilized. Wilchek et al.³¹¹⁾ have summarized various conditions that have been employed to elute proteins from immuno-affinity columns. These include low and high pH, urea and chaotropic salts. The effect that these agents will have on protein activity must always be considered prior to any attempt at immunoaffinity chromatography.

9 Optimizing Preparative-Scale Isolations

The development of a scale-up strategy follows several well-defined guidelines based upon the amount of sample involved, the particle size of the adsorbent and column dimensions²³⁰⁾. These are summarized in Table 3. First, the purification steps are

Table 3. Step-by-step optimization and scale-up strategy

Step	Sample amount	Particle size	Column dimensions
Optimization of chromatographic conditions	1-10 mg	5-10 μ m	3-8 mm D 7.5-30 cm L
Comparison of analytical and preparative particles	1-10 mg	50-100 μ m	3-8 mm D 7.5-30 cm L
Determination of maximum mass load	10-100 mg	50-100 μ m	3-8 mm D 7.5-30 cm L
Scale-up	1-100 g	50-100 μ m	5-15 cm D 20-50 cm L

D, diameter; L, length

optimized on an analytical scale to achieve the maximum degree of purity of the protein of interest. With regards to HPLC techniques, such micropreparative separations are generally performed with less than 10 mg of sample using columns with internal diameters of 3 to 8 mm and lengths of 7.5 to 20 cm and packed with particles of sizes 5 to 10 μ m. Parameters that can be changed to achieve optimum resolution include gradient conditions, strength of organic solvent, flow rate and temperature. Next, the analytical separation is repeated using an identical sample size and the same analytical column but packed with particles to be used in the preparative-scale isolation, for example those with sizes of 50 to 100 μ m. This allows one to confirm that the separation parameters achieved with the analytical and preparative-size packings are similar, particularly with respect to column selectivity. The third step in the scale-up process is to increase the sample size and determine the mass load level of the preparative-size particles in the analytical column. This is achieved by resolving progressively larger

from agar, is not commonly used in protein electrophoresis because of its poor sieving (resolving) properties and fragility. Polyacrylamide is formed by the polymerization of acrylamide monomers that are covalently crosslinked and has become the matrix of choice for resolving protein mixtures on an analytical scale. The major limitation that has hampered the scale-up of PAGE has been the relatively small amount of material that can be accommodated by the standard apparatus. As detection limits of peptides and proteins are continually reduced, however, PAGE is increasingly becoming a powerful tool to achieve their purification, particularly for primary structural investigations. Initially, proteins in polyacrylamide gels were visualized by staining with Coomassie blue which is sensitive to about 1 µg of protein. Newer staining techniques utilizing silver or nickel^{231,232} provide sensitivity to approximately 10 ng of protein. The most sensitive detection is produced by autoradiography where radiolabeled proteins are visualized after placing the gel in contact with x-ray film. The remainder of this section will briefly summarize recent advances in PAGE, i.e. SDS-PAGE, isoelectric focusing and two dimensional PAGE.

10.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS, an anionic detergent, binds to proteins in a manner that overwhelms their individual charge and induces a conformational change to rod-like structures. As a result, all proteins possess an equal charge density per unit length such that electrophoretic migration is determined by molecular weight. SDS-PAGE is performed using either a continuous buffer system²³³ or, more commonly, under discontinuous conditions such as those described by Laemmli²³⁴, Neville²³⁵ and Wyckoff et al.²³⁶ The latter case is portrayed in Fig. 12. A stacking gel containing a large pore po lyacrylamide overlays a separating gel with a more restrictive pore size. Both gels

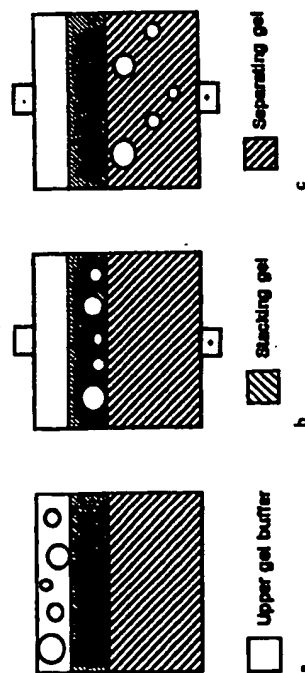


Fig. 12a-c. The principle of discontinuous electrophoresis in the presence of SDS. a. Proteins are treated with a reducing agent and SDS to dissociate multimers into their constituent polypeptide chains with similar charge to mass ratios. b. When an electric field is applied, the polypeptides migrate into the stacking gel between two different pH buffers forming a sharp zone at the interface of the separating gel. c. The polypeptide chains enter the separating gel where they are resolved according to their molecular weight.

Table 4. Useful protein molecular weight standards

Protein (source)	Approximate molecular weight (Da)	Supplier
Blue dextran 2000	2,000,000	PL, S
Thyroglobulin (cow)	670,000	B, S
Urease, hexamer (jack bean)	575,000	S
Ferritin (horse)	440,000	C, P, PL, S
β-Gluconomerase	290,000	C
Urease, trimer (jack bean)	272,000	S
Catalase (cow)	232,000	P, PL
β-Amylase (sweet potato)	200,000	S
Myosin (rabbit)	200,000	B, D, L, S
α ₂ -Macroglobulin (human)	180,000	S
RNA polymerase, β'-subunit	165,000	P
Aldolase (rabbit)	158,000	C, P, PL
γ-Globulin (cow)	150,000	B
RNA polymerase, β-subunit	155,000	P
β-Galactosidase (<i>Escherichia coli</i>)	116,000	B, D, S
Hexokinase	100,000	C
Phosphorylase B (rabbit)	97,400	B, D, L, S
Fructose-6-phosphate kinase (rabbit)	84,000	S
Ovotransferrin (chicken)	78,000	PL
Hemocyanin (<i>Limulus polyphemus</i>)	70,000	S
Serum albumin (cow)	66,200	A, B, C, D, L, P, PL, S
Pyruvate kinase (chicken)	58,000	S
Glutamate dehydrogenase	55,000	D
Fumarate (pig)	48,500	S
Ovalbumin (chicken)	45,000	A, B, C, D, L, P, PL, S
Alcohol dehydrogenase	39,000	D
RNA polymerase, α'-subunit	39,000	P
Glyceroldehyde-2-phosphate dehydrogenase (rabbit)	36,000	S
Lactate dehydrogenase (rabbit)	36,000	D, S
Pepsin	34,700	C, S
Carbonic anhydrase (cow)	30,000	B, C, D, S
Triosephosphate isomerase (rabbit)	26,600	S
Chymotrypsinogen A (cow)	25,000	C, L, P, PL
Trypsin inhibitor (soybean)	21,500	A, B, D, P, S
β-Lactoglobulin B (cow)	18,400	L, S
Myoglobin (horse)	17,200	B, D
Hemoglobin (cow)	16,000	S
Lysozyme	14,400	B, C, L, S
α-Lactalbumin (cow)	14,200	S
Ribonuclease A	13,700	PL
Cytochrome C (horse)	12,400	A, C, D, P, S
Aprotinin (cow)	6,500	A, S
Trypsin inhibitor (cow)	6,200	L
Insulin, alpha and beta chains	3,000	A, D, L

Table 4. (continued)

A:	Amerham Corporation, Arlington Heights, IL;
B:	BioRad Laboratories, Richmond, CA;
C:	Calbiochem, San Diego, CA;
D:	Diversified Biotech, Newton Centre, MA;
L:	Life Technologies, Inc, Gaithersburg, MD;
P:	Pierce Chemical Company, Rockford, IL;
PL:	Pharmacia LKB Biotechnology, Inc, Piscataway, NJ;
S:	Sigma Chemical Company, St. Louis, MO

as well as the electrophoresis running buffer possess different pH and ion concentrations. As proteins migrate through the stacking gel, they are concentrated in a thin zone between the leading buffer ion of the stacking gel and the trailing ion of the running buffer. Upon reaching the separating gel, protein mobility becomes inversely proportional to molecular weight. Using a set of predetermined molecular weight markers (Table 4) to construct a standard curve of protein mobility, the apparent molecular weight of an unknown can then be calculated. Glycoproteins containing a high percentage of carbohydrate are an exception since they bind to smaller amounts of SDS on a weight basis and thus exhibit reduced gel mobility.

As an analytical tool, discontinuous SDS-PAGE offers a very high degree of resolution necessary to follow a purification scheme. Optimization of the running conditions has permitted sharp resolution of proteins over a molecular weight range of 1,500 to 100,000 Da on a single gel²³⁷. Identification of the molecular weight of impurities that may contaminate the protein of interest allows one to choose an appropriate liquid chromatographic technique to achieve a greater degree of purity. In most instances, however, the very component which makes this useful for analyzing protein homogeneity (i.e. SDS) also renders it impractical as a preparative technique. The notable exception to this limitation applies when the goal of protein purification is a primary structural rather than functional analysis. Proteins resolved on SDS containing polyacrylamide gels can often be recovered by electroelution for direct amino acid sequence analysis via amino-terminal Edman degradation (see below).

10.2 Isotachopheresis

The principle of electrophoretic separation by isotachopheresis is very similar in many respects to that of disc electrophoresis discussed above, in particular the effect of sample ion migration through the stacking gel. Thus, sample components migrate as a zone in an aqueous medium between a leading buffer ion and a trailing ion and are separated from each other in order of their charge differences²³⁹. Since the leading and trailing ions are chosen such that the sample ions have an intermediate mobility between them, a zone sharpening effect tends to counteract the effects of diffusion. Furthermore, addition of intermediate mobility compounds, such as amino acids, to define the boundaries of the individual sample components has resulted in the resolution of isotachopheresis being in many cases equivalent to that of other electrophoretic techniques. As an analytical technique, isotachopheresis provides the ad-

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ditional benefits of small sample requirements and short analysis time. On the preparative-scale, however, its full potential is yet to be realized. Incorporation of on-line detection and fraction collection systems has, in a few cases, enabled isotachopheresis to be utilized as an integral step in protein purification procedures²³⁹. The reader is referred to the review article by Holloway and Battersby²⁴⁰ for further reference.

10.3 Isoelectric Focusing

The migration of proteins in isoelectric focusing (IEF) gels is determined by their pI and the development of a pH gradient which is formed by inclusion of highly mobile amphoteric components (ampholytes) in the gel matrix (Fig. 13). Ampholytes consist

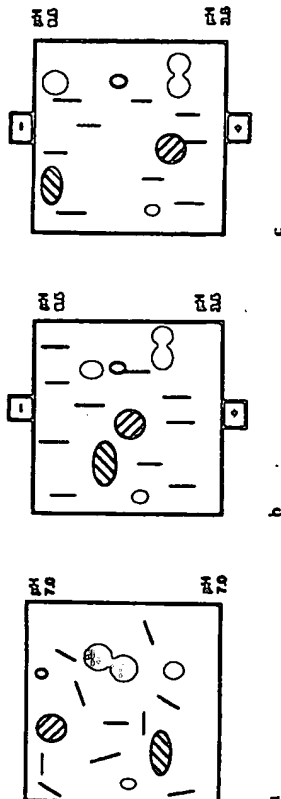


Fig. 13 a-c. The principle of isoelectric focusing. a. Proteins are applied to a gel containing carrier ampholytes (indicated by the small bars). A random distribution of ampholytes produces a uniform pH of 7.0 throughout the gel. b. When an electric field is applied, the ampholytes quickly migrate to their respective isoelectric points forming a pH gradient, illustrated here as extending from pI 3.5 to 9.5. The protein molecules migrate slowly to the pH corresponding to their isoelectric points. c. An equilibrium is reached when the net charge of the proteins is 0 and their migration stops.

of a collection of zwitterionic, small molecular weight polyaminopolycarboxylic acids with differing pIs. In the presence of an electric field, they arrange themselves in a manner such that the most basic are located near the cathode and the acidic near the anode. A focusing effect is achieved when the proteins migrate to the position in the pH gradient where their net charge is zero. Table 5 lists several useful protein isoelectric point standards that can be utilized to estimate the pI of an unknown protein. IEF is primarily a tool for analytical purposes although it can be used for preparative-scale isolations using either a column apparatus with a sucrose gradient to minimize thermal-induced convection currents²⁴¹ or a horizontal electrophoretic chamber²⁴². High resolution preparative IEF has recently been reviewed and this paper can be consulted for further information²⁴³. However, a clear disadvantage relating to the use of IEF in a preparative mode is the tendency of proteins to precipitate out of solution at their pI where they are less soluble than in a charged state.

Table 5. Useful protein isoelectric point standards

Protein (source)	Approximate pI	Supplier
Cytochrome C (horse)	10.60	B, PL
Trypsinogen (cow)	9.30	S
α -Chymotrypsin	8.80	B
Lactate dehydrogenase (rabbit)	8.30-8.55	S
Myoglobin (whale)	8.05	B
Myoglobin (horse)	6.76-7.16	B, S
Carbonic anhydrase (human)	6.50	B, S
Carbonic anhydrase (cow)	5.85-6.00	B, S
Azurin (<i>Pseudomonas aeruginosa</i>)	5.65	PL
β -Lactoglobulin A (cow)	5.13-5.25	PL, S
β -Lactoglobulin B (cow)	5.10-5.35	B, PL
Phycocyanin	4.65	B
Trypsin inhibitor (soybean)	4.55	S
Glucose oxidase (fungi)	4.25	PL
Amyloglucosidase (<i>Aspergillus</i>)	3.55-3.65	PL, S

B: BioRad Laboratories, Richmond, CA;

PL: Pharmacia LKB Biotechnology, Inc, Piscataway, NJ;

S: Sigma Chemical Company, St. Louis, MO

Since mobility in analytical IEF gels is not influenced by molecular weight, it is desirable to utilize a matrix containing nonrestrictive pores. As a consequence, both agarose ²⁴⁴, polyacrylamide gels ²⁴⁵ and granulated gels of the Sephadex or BioGel type ²⁴⁶ have proven to be popular choices. The resolving potential of IEF is tremendous. Proteins with a single charge difference can be separated on an analytical scale ²⁴⁷. In practical terms, this translates into a difference in pI values of 0.001 to 0.01. In many cases this is a clear advantage over SDS-PAGE allowing the detection of impurities following size exclusion chromatography, for example. It must be cautioned, however, that charge heterogeneity of proteins can also be produced by post-translational modifications including phosphorylation ²⁴⁸ and glycosylation ²⁴⁷. A certain degree of charge heterogeneity due to these effects may often be tolerated in a protein preparation when the objective of the purification protocol is, for example, to prepare an antigen for immunization or primary structural analysis ²⁴⁹.

10.4 Two-Dimensional Polyacrylamide Gel Electrophoresis

Both SDS-PAGE and IEF are each capable of resolving 30 to 40 different proteins on a single gel. Resolution of between 100 and 200 components, however, can be achieved by sequential combination of these techniques ⁴⁶. Commonly, IEF is performed first in a small diameter tube gel which is subsequently layered over a SDS-PAGE slab gel ²⁵⁰. This principle is illustrated in Fig. 14. Separations in the reverse order have also been reported, i.e. SDS-PAGE preceding IEF ²⁵¹. In either case, the handicaps of both SDS-PAGE and IEF when used on a preparative-scale are apparent

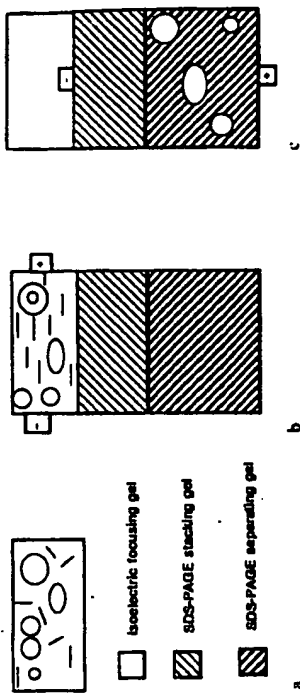


Fig. 14a-c. The principle of two dimensional gel electrophoresis. a. Proteins are applied to an isoelectric focusing gel. Carrier ampholytes are indicated by the small bars. b. The proteins are first separated according to their isoelectric points. The isoelectric focusing gel is then connected to an SDS-PAGE gel at a right angle to the original separation. c. The polypeptide chains are finally resolved in the separating gel according to their molecular weight

in two-dimensional PAGE. The great utility of this technique lies in its application to monitoring a purification scheme. For instance, identification of a contaminant with the same pI but different molecular weight as the protein of interest directs one to use ultrafiltration or size exclusion chromatography as the next purification step. In contrast, if the impurity possesses a different pI but similar molecular weight, ion exchange chromatography can be used at the next step. A contaminant of similar charge and molecular weight as the protein of interest suggests the use of a hydrophobicity-based separation step, such as reversed phase or hydrophobic interaction chromatography. Like SDS-PAGE, two-dimensional PAGE is limited in its applicability to isolation of proteins for amino acid sequence analysis and antibody production. The former was discussed above. Both monoclonal ²⁵² and polyclonal ²⁵³ antibodies have been prepared using proteins excised from Coomassie blue or silver stained polyacrylamide gels. Since such proteins are denatured, however, the antibodies that are produced are frequently directed against linear rather than conformational determinants. One of the best methods of detecting their specificity is the use of immunoblotting (Western blotting) procedures whereby proteins separated by one or two dimensional PAGE under denaturing conditions are electroblotted to nitrocellulose membranes and reacted with the antibodies ²⁵⁴.

11 Amino Acid Sequencing of Proteins

11.1 Micro-Sequence Analysis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate can resolve complex mixtures of polypeptides. Electrophoretic elution from gel slices or electrophoretic transfer to nitrocellulose or glass fiber membranes is used to recover small quantities of proteins from SDS-PAGE gels for peptide mapping and protein

sequencing. Proteins can be resolved on two-dimensional polyacrylamide gels, presure extracted onto short HPLC reversed phase columns and then collected for direct sequencing^{23,25-27}. Sequence information from very small quantities of proteins electroeluted from discontinuous SDS polyacrylamide gels²⁸ or electroblotted onto polyvinylidene difluoride membrane²⁹ or on glass fiber filter^{25,26} is routinely obtained and used for synthesizing nucleotide probes for isolation of genes. Current methods of gas phase sequence analysis require a minimum of 5 to 10 picomoles of protein^{26,1}. For an average protein of 10,000 to 50,000 Da, this represents from 0.05 to 0.5 µg of material, well within the sensitivity of detection of proteins in polyacrylamide gels using present staining technology. Samples from HPLC separations are collected directly. By optimizing the instrument and reduction of column internal diameter, detection can be improved more than tenfold^{24,1}. Future developments in amino acid sequencing are predicted to decrease the amount



Fig. 15. Purification and N-terminal amino acid sequence analysis of a viral protein derived from a polyprotein precursor. Human lymphocytes were infected in vitro with the human immunodeficiency virus type 1 (HIV-1), cultured in medium containing [35S]methionine plus [3H]valine or [3H]leucine and lysed in detergent solution. Viral proteins were recovered by immunoprecipitation, resolved by SDS-PAGE and visualized by fluorography. Specific immunoprecipitation of viral proteins was determined by comparison of those recovered using a normal human serum negative control (left lane) with those precipitated by an antiserum from an individual infected with HIV-1 (right lane). The migration of molecular weight markers is indicated on the left in kDa. A protein of 24,000 Da (indicated by arrow) was cut out of the gel, recovered by electroelution and its amino acid sequence determined (lower right). Based upon the radioactive amino acids identified (indicated by asterisk), the N-terminus of this protein (designated p24) was localized to residue 133 of the 512 amino acid open reading frame previously demarcated by DNA sequencing (upper right). The DNA sequence of this segment is given in lower case letters below the amino acid sequence. The N-termini of the p7 and p9 proteins isolated on other SDS gels were similarly localized to residues 378 and 449, respectively.

of protein required to the attomole range^{26,31} enabling SDS-PAGE, particularly in a two dimensional format, to become an extremely powerful protein purification technique.

11.2 Radiochemical Sequence Analysis

One modification of this procedure that is currently available to achieve a comparable level of sensitivity is the use of radioactively labeled amino acids in the purification and sequence analysis of proteins synthesized by cells in tissue culture. [35S]-labeled methionine or cysteine either alone or in combination with [3H]-labeled amino acids are metabolically incorporated into cellular or viral proteins which can subsequently be resolved on one or two dimensional SDS gels, isolated by electroelution and directly analyzed by N-terminal Edman degradation^{26,4-26,6}. Figure 15 illustrates how this technique has been utilized to identify natural proteolytic cleavage sites in viral polyproteins whose DNA sequences have previously been established.

12 Recombinant DNA in Protein Overproduction

12.1 Prokaryotic Expression

As a result of the advent of recombinant DNA technology the list of genes for numerous protein products of pharmaceutical and industrial importance is growing rapidly. The technology for cloning and sequencing of genes is better perfected than purifying and characterizing their protein products. Most proteins can be purified in microgram amounts by using fast protein liquid chromatography, high-performance liquid chromatography, affinity chromatography or elution from SDS polyacrylamide gels and renaturation. However, some proteins need to be produced in kilogram quantities and homogeneous purity to meet industrial needs. Expression of cloned genes was originally developed in bacterial systems but has since been accomplished in insect and eukaryotic host cells as well. Plasmids pKc30 and pKc31 have been used for large-scale bacterial expression of cloned genes^{26,7}. In plasmid pKc31, the pL promoter originally from phage is kept repressed by a thermolabile repressor, CITS 857. The cells are usually grown to mid-log phase and then heat is used to induce the synthesis of the cloned protein under the control of the pL promoter^{26,8}. Construction of an overexpressing strain permitted for the first time purification to homogeneity of chorismate synthase from *E. coli*^{26,9}.

12.2 Eukaryotic Expression

A major disadvantage in using bacterial host cells for expression of recombinant proteins concerns the fact that prokaryotic translation systems do not recognize eukaryotic post-translational signals such as sites of glycosylation, phosphorylation and signal peptide cleavage. Insect and eukaryotic host expression systems, in contrast, produce recombinant proteins containing such post-translational modifications. The recombinant baculovirus vector has been the most widely utilized for expression of cloned genes in insect cells^{27,0,27,1}. Recently, a protein-free medium that is capable of supporting large-scale insect cell culture, virus propagation and cloned gene ex-

pression has been developed to enhance the purification of recombinant proteins²⁷². Eukaryotic viruses have been modified to produce vectors for cloning, selection and expression of heterologous genes. Examples of these include vaccinia virus, simian virus 40, adenovirus and a variety of retroviruses. Proper post-translational modifications and processing of recombinant proteins produced in these systems has been documented²⁷³. Several review articles on this subject have appeared and may be consulted for further reference²⁷⁴⁻²⁷⁶.

Rational alteration of enzyme properties using site-directed mutagenesis of cloned genes is being examined using a variety of model systems and these have been recently summarized²⁷⁷. Single amino acid substitutions have produced mutant enzymes from *Bacillus amyloliquefaciens* with increased activity, altered specificity and altered pH activity²⁷⁸. Changing an amino acid in the active site of an enzyme also alters its substrate interactions. Amino acid substitutions in *B. thermoproteolyticus* subtilisin at positions 131 and 172 were shown to decrease the rate of thermal inactivation via a mechanism involving enhanced Ca^{+2} ion binding²⁷⁹.

13 Fusion Proteins

13.1 β -Galactosidase Fusion Proteins

Several *E. coli* cloning vectors in which DNA fragments can be cloned into the β -galactosidase (*lacZ*) gene via restriction sites are available. The protein coded by the inserted DNA fragment is expressed as a chimeric β -galactosidase fusion protein (Fig. 16). This technique offers several advantages such as ease of screening of recombinants, ease of purification and enhanced stability of the foreign gene product in the host cells. A variety of promoters have been used to express the fusion proteins at significant levels. A popular double-stranded bacteriophage, lambda *gt11*, is used for inserting DNA near the 3' (C terminus) end of the *lacZ* gene. 53 base pairs upstream from the translation termination codon^{280,281}. Monoclonal antibodies which recognize native β -galactosidase as well as N- or C-terminal β -galactosidase fusion proteins are commercially available. These antibodies can be used to purify the recombinant proteins by immunoaffinity chromatography and the fusion proteins subsequently used for immunization to prepare an antiserum from which the antibodies against the foreign gene product can be isolated by passage through a β -galactosidase affinity column²⁸². β -Galactosidase fusion proteins have also been purified by binding to and elution from aminophenylthiogalactosidyl-Sepharose columns²⁸³.

13.2 Other Fusion Proteins

Several notable handicaps have been encountered with the production and purification of fusion proteins using the techniques described above. First, since the added protein segment is quite large, the foreign gene product often does not properly fold into a native, active conformation²⁸⁴. Denaturation followed by slow renatur-

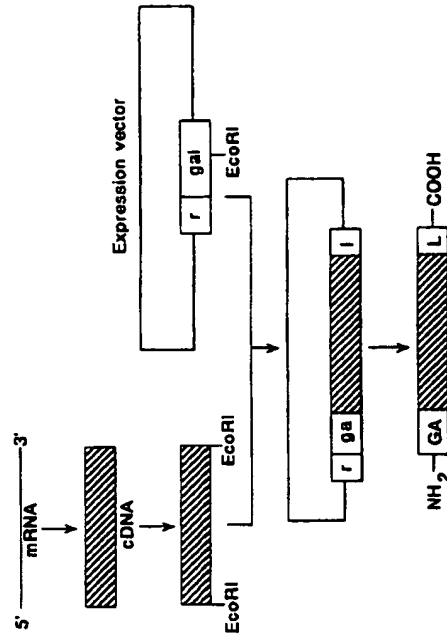


Fig. 16. Expression of a foreign gene as a β -galactosidase fusion protein. The foreign gene is first synthesized as cDNA from mRNA and suitable restriction endonuclease sites (e.g. *EcoRI*) generated at each end. The cDNA (hatched box) is then introduced into the homologous restriction endonuclease site in the β -galactosidase gene (*lacZ*) of the expression vector (open box). The *lacZ* gene is preceded by the necessary regulatory DNA sequences required for gene expression, e.g. start codon (ATG), promoter (P), operator (O) and ribosome binding site (r). Note that the DNA sequence of the *lacZ* gene is disrupted by the cDNA. Expression of the hybrid gene after transformation of bacterial host cells result in a fusion protein containing the cloned protein inserted into the β -galactosidase protein and terminated at the stop codon (TGA) of the cDNA or *lacZ* gene. Unlike the normal β -galactosidase protein, the fusion protein is unable to hydrolyze synthetic substrates enabling selection of bacterial cells containing the fusion protein by a color reaction.

ation may be required to obtain a native state of the introduced polypeptide²⁸⁵. Second, the high binding affinities of most monoclonal antibodies and polyclonal antisera against the host protein segment necessitates use of severe denaturants for elution from immunoaffinity columns. Finally, once the fusion protein has been purified, it has often been difficult to remove the added protein from the desired polypeptide. Attempts to circumvent this problem have employed chemical²⁸⁶⁻²⁸⁹ or enzymatic^{284,290,291} methods. Recently, the use of a short, hydrophilic peptide engineered into the N-terminus of fusion proteins has solved many of these problems²⁹¹. This peptide was less than 10 amino acids in length and did not interfere with the folding and subsequent biological activity of the desired proteins. Furthermore, an antipeptide antiserum can be prepared for immunoaffinity purification using the free peptide to elute bound fusion protein under physiological conditions. Engineering the proper peptide sequence in the fusion protein allows its removal by proteolytic enzymes of appropriate specificity. Alternatively, use of a lysine-rich peptide has permitted biotinylation of the recombinant protein without loss of biological activity such that it may serve as a ligand for affinity purification of a variety of receptors on avidin-containing supports²⁹².

When proteins are overexpressed in *E. coli*, some of them accumulate as insoluble aggregates known as inclusion bodies. Sometimes the formation of inclusion bodies

can be prevented by changing the media and growth conditions of *E. coli*. Proteins can also be engineered so that they are secreted out of the cell. However, many proteins are extracted from the cells and renatured to gain biological activity. The protein contained in inclusion bodies may be solubilized by treatment with chaotropic agents, acids or alcohols.

14 Future Developments

The future of protein purification rests on the development of new products as well as innovative technologies of microanalytical separations. In the former category are fluoropolymer supports for immobilization of affinity ligands. Covalent attachment of a perfluoralkylating chain to primary amino groups of proteins allows them to be strongly adsorbed to polyfluorocarbon-based supports in either a solid or liquid phase²⁵³. Continuous liquid-liquid extraction based upon this principle may soon become common industrial practice to purify recombinant proteins from spent media. A similar type of liquid-liquid extraction occurs in centrifugal partition chromatography, a new application based upon the distribution of solute mixtures between two liquid phases in the absence of a solid support²⁵⁴. Unlike traditional counter-current partition chromatography, this technique utilizes separation columns connected in series within cartridges arranged around a centrifuge rotor. The mobile phase, which is often the less dense of the two immiscible solvents, is pumped through the columns where, in droplet form, it comes in contact with the stationary phase which is maintained in the system by centrifugal force. Another novel application soon to become commercially popular is radial flow liquid chromatography. Instead of the conventional mode of applying a sample to the top of a column and separating it with downward axial flow, the sample is distributed around the outer circumference of the column and separated by radial flow (Fig. 17). The capacity of the column can therefore be increased by a factor proportional to the length of the column. Capillary

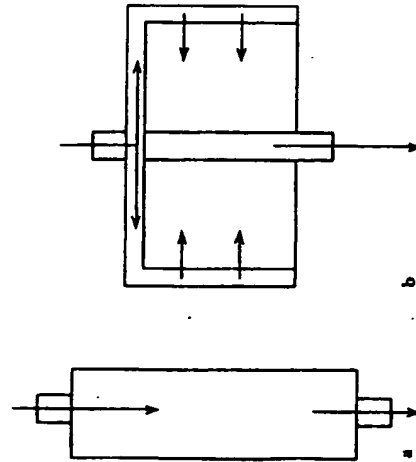


Fig. 17a, b. The principle of radial flow chromatography. a. Conventional tubular liquid chromatography with downward solvent flow. b. Radial flow chromatography employs solvent flow from the outer circumference of the column inward to a central collection tube

electrophoresis and open tubular liquid chromatography represents promising new methods of analytical protein separation utilizing sample sizes on the nanoliter scale²⁵⁵. Fused silica capillaries with internal diameters of less than 100 μm and wall thicknesses of less than 200 μm allow efficient dissipation of heat to permit extremely high resolution separations. Furthermore, the high degree of purity of silicone dioxide used in manufacturing these capillary tubes provides a very inert surface for improved chromatography of bioactive proteins. Capillary electrophoresis can be performed in a variety of different types of media, including aqueous or organic solutions or capillaries filled with agarose or polyacrylamide. A recent review has compiled a list of both standard and unknown proteins that have been separated by this method²⁵⁶, although more sensitive detectors need to be developed before its full potential is realized. Open tubular liquid chromatography also utilizes long, thin capillaries but to which a stationary phase such as that used in reversed phase chromatography (e.g. C-18) has been coated onto the inner wall surface. Again, the major obstacle that needs to be overcome before this technique becomes more applicable to the protein chemistry laboratory is the development of sensitive methods of detection. Once this and other obstacles are overcome, future protein separations will be capable of analyzing single cell volumes on a routine basis.

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